ASPECTS OF THE TRANSDERMAL PERMEATION AND ANALYSIS OF BETAMETHASONE 17-VALERATE

A Thesis Submitted to

RHODES UNIVERSITY

in Fulfilment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

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October 1987

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ABSTRACT

The current world-wide interest in transdermal drug delivery makes the prospect of valid *in vitro* diffusion cell methodology highly attractive. A new laboratory diffusion cell has been designed and constructed based on theoretical principles and practical permeation reports surveyed in recent literature, and has been applied to the monitoring of betamethasone 17-valerate permeation. The cell performance has been validated with respect to hydrodynamic mixing efficiency and temperature of the receptor phase. The steady-state permeation of this corticosteroid has been monitored through various synthetic and animal membranes in order to select the most appropriate media for *in vitro* study. The permeation of betamethasone 17-valerate has been monitored from various types of commercial and extemporaneously prepared semisolid topical formulation (cream, lotion, ointment and scalp application), through silicone membrane, human and weanling pig stratum corneum, and full thickness hairless mouse skin, and these *in vitro* results have been compared to data from *in vivo* blanching assays, using the same formulations, in an attempt to correlate the findings.

This experimental methodology has necessitated the development of ancillary analytical techniques. A column-switching high-performance liquid chromatographic method has been developed for the rapid on-line clean-up and analysis of betamethasone 17-valerate contained in the various topical formulations, which minimizes sample handling and extraction procedures. The method has been modified for the analysis of this corticosteroid in the isopropyl myristate receptor phase used in the *in vitro* permeation experiments, and scintillation counting of tritium-labelled water has been used to verify the integrity of the animal membranes.

The comparison of *in vitro* permeation and *in vivo* blanching results indicate good correlation of the data in certain instances. The closest correlations have been observed when the human stratum corneum has been used in vitro and these results are compared to data from the occluded mode of the blanching assay. The results of the porcine and murine media have also correlated with the human *in vivo* data, whereas the silicone membrane appears applicable only in certain *in vitro* experiments. The results indicate that valid, comparative percutaneous absorption data may be obtained *in vitro* by using a well designed, validated diffusion cell system.

ACKNOWLEDGEMENTS

It is with sincere gratitude that the following acknowledgements are made:

My supervisor, Professor J M Haigh, who willingly gave of his time, advice, encouragement and research grant whenever they were requested; and for having had the courage to allow me to independently persue research along lines I considered important.

Professors I Kanfer and H Parolis, and the staff of the School of Pharmaceutical Sciences for their assistance, advice, and for the use of departmental facilities.

Leon Purdon and Owen Campbell for their friendship, technical expertise and assistance which was rendered whenever it was requested.

Eric Meyer, the blanching trial student volunteers and the skin donors for giving their time and of their bodies in the interests of this research.

Jenny Purdon and Maureen James for proof-reading this manuscript and for their invaluable advice.

The glassblower, Mr J Murray, for constructing the permeation cells.

Mr R Cross and the staff of the Electron Microscopy Unit for their examination of the skin specimens.

The CSIR and the H Bradlow Foundation for their generous financial assistance.

My peers in the biopharmaceutics laboratory for their interest, friendship and for the laughter that kept us all sane during the endless days and nights.

Finally, Karen for her love, encouragement and help during the four years preparing this thesis.

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1. THEORY OF TRANSMEMBRANE DIFFUSION

This study is concerned with drug absorption through the skin, and especially with the development of a laboratory system that would adequately simulate the absorption process for a particular corticosteroid. It is appropriate, therefore, that the investigation should commence with a brief review of skin structure and the kinetic events that occur as a molecule traverses this membrane. Thereafter, the physical factors or chemicals that may enhance drug absorption and the hydrodynamic problems associated with the use of *in vitro* diffusion cells are summarized as an introduction for the main diffusion cell and membrane theory to follow in Section 2.

1.1 BASIC HISTOLOGY AND DIFFUSION THROUGH THE SKIN

The skin serves several protectional and regulatory functions vital to the homeostasis of the body. It protects the delicate internal organs from ambient influences of insolation, foreign chemicals and invasion by micro-organisms, often being damaged physically or chemically but has a remarkable capability of self-repair. The skin regulates temperature, participates in blood pressure maintenance while preventing fluid loss by its impervious nature, and generally acts as a portal of communication with the outside world via its innervated network. It is not surprising, therefore, with these diverse roles to fulfil that the skin is heterogeneous in structure (Figure 1). The skin may be divided into two main tissue groups, the lower dermis, comprising connective tissue surrounding the blood vessels and glands, and the upper, avascular epidermal strata, the two layers separated by proliferative tissue.

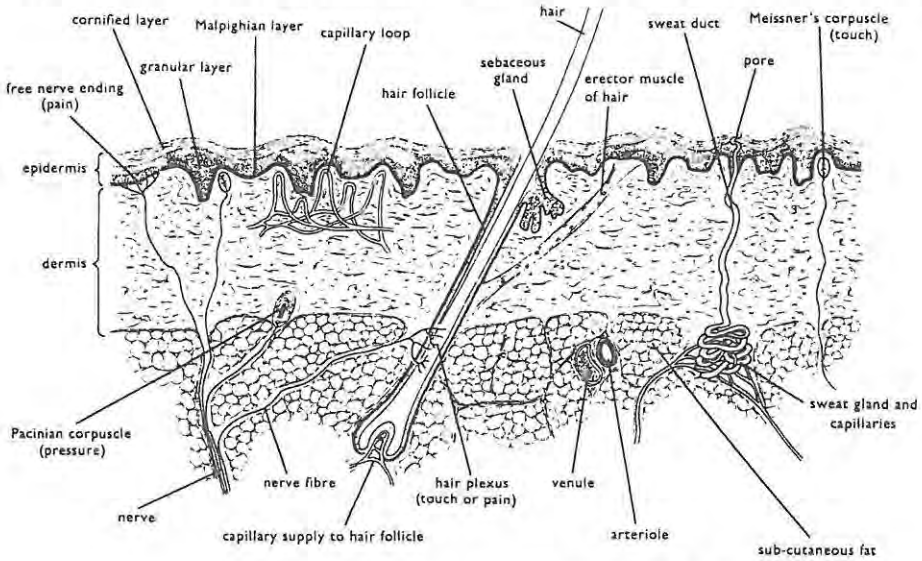


FIGURE 1: Generalized structure of the skin.

The epidermis is not a flat sheet, thickened ridges of epidermal cells (rete ridges) undulate into the underlying dermal layer. The epidermis varies in thickness at different anatomical sites, corresponding to the degree of abrasive trauma suffered by the skin at each site, and the layer is penetrated by the hair follicles and ducts of the exocrine glands that have their source in the dermis. The dermo-epidermal junction separates the generative basal layer of the epidermis from the underlying dermis. This region of adherence of the two strata provides a certain degree of support for the epidermis and controls the passage of certain endogenous and exogenous moieties. This junction may readily be cleaved by the action of heat or other chemical means if the strata are to be separated for experimental work.

The basal layer cells are active metabolically and their mitotic division replaces the dead cells lost from the exposed horny layer. The basal layer also contains the melanocytes that produce the melanin pigment which protects the underlying strata against radiation damage. The newly divided cells undergo specialization as they migrate from the generative basal layer to the superficial stratum corneum, altering histologically, flattening in shape and undergoing regression of their nuclei and other organelles with the progressive deposition of keratin filaments. These cells are held together by desmosomes that maintain the integrity of the epidermal layer but allow oxygen, nutrients and larger cells (such as melanocytes or leukocytes) to pass through the intercellular fluid. Fibrous keratin molecules are actively synthesized by migrating keratinocytes in the granular layer of the epidermis. This layer divides the metabolically active cells of the basal layer and the dead, keratinized cells of the horny layer.

Approximately 10–15 layers of dead, flattened, fully keratinized cells form the impervious, outer stratum corneum, the cells overlapping to form continuous sheets without intercellular pores. It is the selective permeability of these highly organized cell layers that protects the body from invasion by environmental chemicals. The cells are continuously abraded from the skin surface and replaced from the dividing basal layer, the progression from mitosis to shedding taking approximately 28 days.

The strength and flexibility of the epidermis is dependent on the condition of the stratum corneum, which in turn is controlled by its relative content of lipid, protein and hydrophilic substances. The correct water balance of this tissue is imperative to the maintenance of suppleness and elasticity as the water molecules interact with the keratin filaments within the cells separating these protein molecules, and causing the entire horny layer to swell appreciably.

The dermis is much thicker than the epidermis and consists mainly of proteinaceous connective tissue (mainly collagen and elastin) and mucopolysaccharide (mainly hyaluronic acid). This matrix is traversed by blood vessels, nerves and lymphatic structures, and penetrated by the eccrine glands and hair follicles. The dermis has a copious blood supply which delivers nutrients, oxygen and immunological agents to the skin strata and appendages, and removes catabolic waste products. The constriction or dilation of these vascular elements also controls the temperature of the body and assists in blood pressure regulation. Exogenous chemicals that diffuse through the stratum corneum and epidermis are rapidly absorbed by the dermal vasculature and cleared from the skin via the systemic circulation. This rapid clearance does not allow diffusant to accumulate in the dermis and, thus, sink conditions are maintained as the driving force for further passive diffusion. The patency of these blood vessels may have important consequences for the study of drug absorption. Corticosteroids, for example, have a vasoconstrictive side effect, or the stenosis experienced in thermoregulation may restrict local blood flow and thereby decrease the clearance of topically applied agents, thereby deviating from sink diffusion conditions. Vasodilation, on the other hand, may clear diffusant molecules faster, and hence a difference in the *in vivo* response may be experienced at different ambient temperatures.

Equally important from an investigative viewpoint is the fact that the dermis and lower epidermal cell layers are metabolically active and may catabolize exogenous chemicals before they are absorbed into the systemic circulation. This requires consideration when topically applied drugs are intended for systemic action or if percutaneous absorption is monitored by serum drug concentrations.

The numerous appendages that originate in the dermis and penetrate the epidermal layers may provide facile portals for drug absorption. The eccrine sweat glands comprise a secretory coil in the lower dermal layers and a duct which extends through the dermis and epidermis forming a pore at the skin surface. These glands secrete a weakly ionic, watery solution which regulates the temperature of the body and may act as a solvent for certain hydrophilic chemicals. The hirsute anatomical regions of the body have large apocrine sweat glands, the ducts of which open into the hair follicles. These glands secrete a complex biochemical fluid consisting of lipids, proteins and sugars but, because of their sparse distribution, are not believed to influence percutaneous absorption to any significant extent. The hair shafts that penetrate the dermal and epidermal layers are contained within hair follicles

that are lined by layers of epidermal cells. The epidermis invaginates to enclose the shaft and dermal papilla. The erector pili muscle attaches the inclined follicle to the dermo-epidermal junction and there are usually sebaceous, and possibly apocrine, gland openings into the follicle above this attachment site. The anatomical distribution of hair follicles is non-uniform with the greatest density found in the scalp region. This may bear consideration for agents absorbed mainly through the hair follicles, the degree of absorption per unit area of scalp would be greater than that through other skin sites.

The anatomical distribution of the sebaceous glands is also non-uniform, having the greatest density in the skin of the face and back. The glands usually open into the hair follicles but may emerge directly onto the skin surface in certain parts of the body. The secreted sebum is a highly complex mixture of lipophilic fatty-acid components derived from the catabolism of cellular constituents within the large sebaceous glands; this secretion contributes to the acid mantle of the skin.

The route of skin penetration followed by an exogenous molecule may follow several paths. Initial contact of the prospective diffusant would normally occur with the acidic sebum layer (a mixture of sebaceous and eccrine gland secretions, bacteria and other environmental debris) which forms a cover of varying thickness over the stratum corneum. This layer has negligible barrier potential and does not influence the rate of absorption to any significant degree.

Thereafter the molecule may diffuse along three parallel paths: across the intact stratum corneum (trans- or intercellular), via the hair follicles and adjoining sebaceous glands, or via the eccrine sweat glands. Although the diffusion of polar electrolytes and large molecules with low diffusion coefficients may be faster through the appendageal routes than across the horny layer, the relative scarcity of these appendages in relation to the surface area of the continuous stratum corneum dictates that these paths cannot contribute appreciably to the overall steady-state absorption process. However, initially drug absorption may be faster through these shunt routes than through the intact horny layer and their contribution is therefore important for short intervals after contact of skin with diffusant. With the onset of steady-state, the magnitude of the diffusion through the cells of the stratum corneum surpasses that through the appendages. There are, therefore, two paths of absorption that may predominate at different times and may be represented by a biphasic permeation pattern. This factor may be important for *in vivo* assays that monitor a pharmacological response, such as

vasoconstriction or vasodilation. These responses may be initiated by the molecules permeating rapidly through the shunt appendages and visual observations would not reflect the mass of drug permeating through the barrier layer.

The diffusing molecule passes through three main cell layers, in series, on its path from the skin surface to the dermal capillaries: the stratum corneum, the epidermis and the dermis. The overall resistance to diffusion would be approximated by the sum of the individual resistances of the three strata. Diffusion in the upper dermis and lower epidermis occurs readily through the highly hydrated environments, probably occurring through water-filled intercellular spaces. Once the diffusant crosses the dermo-epidermal junction it is removed by the microcirculation. In contrast, diffusion in the stratum corneum occurs through a relatively nonaqueous, molecularly fibrous environment of the keratinized cells and diffusant translocation ensues with a correspondingly greater degree of difficulty.

The situation becomes complex if the exact location of the rate-limiting resistance to diffusion is sought in the stratum corneum . Diffusant molecules may move intracellularly through the keratinized cells or through the lipoproteinaceous intercellular spaces. The surface area for diffusion through the latter path is relatively small, suggesting that the bulk of the diffusive movement occurs through the cells. If this is the case then the thickened cell walls or, more probably, the intracellular filament-keratin matrix are the logical barriers to diffusion. However, there is no single cell stratum that forms the barrier, the entire horny layer is relatively homogeneous in its resistance to the passage of diffusants. While this superficial layer is dead and diffusion proceeds entirely by passive mechanism, it does have the ability to adsorb diffusant molecules and thereby form a reservoir of the drug which can slowly be depleted over several days following skin contact with the diffusant.

The greatest barrier, therefore, to the absorption of chemicals (both hydrophilic and lipophilic) through the skin, and to the loss of endogenous fluids, are the dead cell strata of the horny layer. The barrier potential of this layer usually controls the rate of permeation of all chemicals entering the body. The epidermal and dermal tissues are metabolically active and may catabolize some of the diffusant but these strata present substantially less of a barrier to absorption than the horny layer. The more "aqueous" cell layers of the lower epidermis and dermis may render some barrier to the absorption of hydrophobic moieties, but this is believed to play a minor role in controlling absorption.

1.2 KINETICS OF THE DIFFUSION PROCESS

The percutaneous drug diffusion process is passive in nature, requiring a concentration differential as the driving force and each molecule requires kinetic energy to effect a nett movement down this gradient. The permeation of a molecule through a membrane, either biological or synthetic, *in vivo* or *in vitro*, would normally occur as follows:

1. The drug must diffuse through the vehicle in which it is contained to the membrane interface and must partition from the vehicle into the upper lamina of the medium.
2. The molecule must diffuse within the membrane, equilibrating laterally and must emerge, eventually under steady-state conditions, from the distal surface of the medium. Adsorptive interaction may be extensive in this layer, forming a reservoir of the molecule. The molecules may be catabolized or degraded in transit through the stratum dependent on the enzymes present or the physical conditions.
3. The molecule then partitions either into neighbouring membrane strata or into the receptor fluid under the influence of the concentration gradient, and adsorption or degradation may occur once again. Diffusion through any one of the layers or any of the partitioning events may control the overall rate of permeation.

It has been established that the skin is a heterogeneous organ composed of several histologically diverse layers, however from the mathematical viewpoint each layer is usually considered homogeneous in its barrier potential. In this way the experimental handling of animal membrane data resembles that for diffusion through homogeneous synthetic media. The steady-state diffusion through a homogeneous medium may be described in terms of Fick's first law which states that the rate of transfer of diffusant through unit area of membrane (the flux) is proportional to the concentration gradient measured normal to the section:

$$J = -D \frac{\partial C}{\partial x} \qquad \text{Equation 1}$$

where J is the flux, D is the diffusion coefficient of the diffusant in the membrane, C is the concentration of the diffusing substance (usually taken as the concentration of the diffusant in the vehicle bathing the donor surface of the membrane), and x is the distance in the direction of the diffusion. The negative sign in the equation indicates that mass transfer is in the direction of decreasing concentration. Fick's first law is only applicable to diffusion in an isotropic medium and where the diffusant concentration at the distal surface of the membrane is zero. While synthetic media may comply with the

isotropic constraint of this equation, the heterogeneous environment of the stratum corneum or epidermis may cause significant deviations from Fickian behaviour. Furthermore, this law is applicable under conditions of steady-state diffusion, a situation that may be generated *in vitro* but is seldom achieved *in vivo*, and any significant degree of adsorption between diffusant and membrane will violate the conditions of applicability.

Hence, several facets of diffusion through anisotropic membranes, such as the skin, violate the conditions for Fick's first law. In these cases the more complex differential equation of Fick's second law is more applicable. This is a fundamental mathematical expression of diffusion which states that the rate of change in concentration with time at a point in the diffusional field is proportional to the rate of change of the concentration gradient at that point. The expanded form of the equation has three components representing the spatial coordinates of diffusion in a cubic volume of the membrane:

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} + \frac{\partial^2 C}{\partial z^2}\right) \qquad \text{Equation 2}$$

where t is the time, and x, y and z are the distances along the three-dimensional diffusion axes. However, for most experimental situations effective diffusion occurs only in one direction through the membrane, that of the concentration gradient, and Fick's second law may be reduced to:

$$\frac{\partial C}{\partial t} = D\,\frac{\partial^2 C}{\partial x^2} \qquad \text{Equation 3}$$

where x is the diffusion distance in the direction of the concentration gradient. The exact nature of this expression will depend on the parameters of the permeation experiment conducted. In most *in vitro* diffusion systems a membrane separates two chambers, one containing an infinite amount of donor vehicle plus drug, and the other containing the receptor medium devoid of drug, or essentially so. A concentration gradient and sink diffusion conditions are therefore established across the membrane. Initially the concentration gradient across the membrane will not be linear as the diffusant equilibrates within the medium, however after sufficient permeation time has elapsed steady-state will be achieved and the effective diffusant concentration at all points in the membrane will remain constant. For an infinite dose of drug applied to the donor surface of a membrane in an *in vitro* diffusion cell, and under sink diffusion conditions, (the permeation situation that has prevailed in this experimental work) Fick's second law has been expanded to the following expression:

$$M = \frac{DC_0 t}{h} - \frac{hC_0}{6} - \frac{2hC_0}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \exp\left(-\frac{Dn^2\pi^2 t}{h^2}\right) \qquad \text{Equation 4}$$

where M is the cumulative mass of diffusant that has passed through unit area of membrane, C_o is the concentration of diffusant in the membrane lamina juxtaposed to the donor vehicle, and h is the thickness of the membrane. This equation is applicable to the typical cumulative mass *versus* time permeation plots that have an initial, nonlinear lag time followed by a linear steady-state plot. As time approaches infinity, this expression may be truncated to the straight line equation:

$$M = \frac{DC_0}{h}\left(t - \frac{h^2}{6D}\right)$$

Equation 5

By differentiating Equation 5 with respect to time an expression is obtained for the steady-state flux (dM / dt) which is the gradient of the straight line:

$$\frac{dM}{dt} = \frac{DC_0}{h}$$

Equation 6

Normally the concentration of the diffusant in the membrane lamina at the donor surface is not known, and cannot readily be measured, however the concentration of the diffusant in the donor vehicle in contact with the membrane can be measured and the following expression is, therefore, more often used:

$$\frac{dM}{dt} = \frac{DCK}{h}$$

Equation 7

where K is the partition coefficient of the diffusant between the vehicle and the membrane. The product of the partition coefficient and the donor vehicle concentration will yield the diffusant concentration in the membrane lamina. The main variables influencing the rate of diffusion are, thus, the partition coefficient and the effective diffusion coefficient, the donor concentration and the membrane thickness. In many cases the dimensions of these variables may be unknown and the equation is simplified to:

$$\frac{dM}{dt} = PC$$

Equation 8

where P is the composite permeability coefficient. The permeability coefficient is often quoted where the diffusion and partition coefficients cannot individ-ually be calculated or where penetration enhancement cannot be directly attributable to either improved diffusion or improved partitioning.

The diffusion coefficient may be estimated if the lag time (L) is obtained from the permeation profile by extrapolating the linear, steady-state portion of the plot to the abscissa where M = 0. If the membrane thickness is known, the diffusion coefficient is then given by:

$$D = \frac{h^2}{6L}$$

Equation 9

Hence, by measuring the steady-state permeation through a membrane, estimating the lag time from this data and knowing the thickness of the membrane, the diffusion coefficient of the solute in the membrane may be calculated. Knowing the concentration of the diffusant in the donor vehicle, the partition coefficient may be estimated from the mass of diffusant that has passed through unit area of membrane, using Equation 8, and the partition coefficient may then be calculated using:

$$P = KD/h$$

Equation 10

Therefore, all the parameters of the steady-state diffusion equation may be estimated from the measured permeation data, if the medium thickness and donor concentration are known. The value of the partition coefficient so calculated should agree with the value obtained by equilibrating the donor vehicle with a section of the membrane and measuring the diminution of solute from the donor phase. The ease of estimation from the permeation plot would favour this method of obtaining the partition coefficient over the physical equilibration experiments.

In certain instances of percutaneous absorption the partitioning characteristics of the permeant between the vehicle and membrane may be highly favourable, generating high drug concentrations within the medium. Alternatively, the barrier potential of the membrane may be minimal or violated by disease, chemical or physical trauma. In these cases the rate of drug diffusion through the medium will be high and the replenishment of the partitioned diffusant at the membrane interface from the bulk vehicle may become the rate controlling step in the permeation process, rather than the resistivity of the membrane itself. Although intravehicle diffusion of drug may not present a problem for mobile solutions, the viscosity of lotions, creams and ointments may greatly hinder re-equilibration.

Higuchi *(1)* has proposed the mathematical relationships that are applicable to this situation in which the membrane is regarded as having negligible resistivity and sink clearance conditions are maintained throughout the permeat-ion time. The rate of permeation would then be governed by the rate of diffusion through the vehicle and is expressed by the simplified equation:

$$M = 2C_0\left(\frac{D_v t}{\pi}\right)^{1/2}$$

Equation 11

where D_v is the diffusion coefficient of the drug in the vehicle and C here represents the initial concentration of diffusant in the vehicle. It is apparent from this equation that the mass of drug permeating to the sink is

proportional to the square-root of the contact time between vehicle and membrane. Permeation of this nature has a characteristic curved profile, exhibiting relatively high flux at early contact times which decreases as the diffusant front regresses into the bulk vehicle, away from the membrane. The path is progressively more tortuous, and takes progressively longer, for drug molecules to diffuse from the region of high concentration in the vehicle to replenish the drug molecules at the membrane interface that have partitioned into the membrane, hence the flux rate decreases with time. This relationship has been observed in the present study for topical formulations that demonstrate relatively high partition coefficients between vehicle and membrane.

Equations 1-11 describe the process of diffusion through an isotropic medium in fairly elementary terms and these mathematical functions have been applied to the analysis of the data from this experimental investigation. Certain assumptions are made in their application: that sink diffusion conditions are maintained throughout the experiment, that extensive adsorption of the diffusant does not occur, and, obviously, that the barrier medium is homogeneous. Several more complex equational models have been reported in the literature *(2-4)* to account for specific diffusional situations, such as the short time approximation method for estimating the diffusion coefficient *(5,6)*. This function is useful if excessively long experimental times are required to establish steady-state diffusion. In these cases the data from the initial, nonlinear period is used in the diffusion coefficient calculation. Full profile analysis, using the permeation data from both the initial nonlinear and steady-state portions of the profile, has also been used *(7)*.

Mathematical expressions have also been proposed to account for the different barriers encountered by a diffusant molecule traversing the epidermal layers of the skin. Here the skin is considered heterogeneous in its composition, however each fraction of its makeup is individually regarded as an isotropic barrier to diffusion. Equations have thus been proposed for complex diffusional barriers in series, for example, as a diffusant would encounter passing from the stratum corneum, through the viable epidermis and into the dermal tissue. The diffusant is also likely to encounter complex parallel pathways, transcellular and transappendageal routes for example. In certain cases the parameters controlling the diffusive process change during the course of the experiment and equations have also been proposed to account for these time-variable conditions. Furthermore, several studies *(8-11)* have proposed pharmacokinetic models for percutaneous absorption that account for drug partitioning into the skin, diffusion through the stratum corneum and

epidermis, and elimination from the systemic circulation and these models therefore require some analysis of drug serum concentrations. These are all relatively complex theoretical manipulations that are beyond the objectives and experimental methodology of this research.

1.3 MODIFYING THE DIFFUSION PROCESS

Many physicochemical factors may influence the rate of chemical permeation through a membrane, both *in vivo* and *in vitro*. Although several of these factors may be controlled in laboratory diffusion experiments, some influences may be unavoidable, or covertly operative, and may thus affect the results obtained in percutaneous absorption studies. In an attempt to elucidate possible mechanisms by which drug penetration may be beneficially modified, or in attempting to correlate data from different experiments, as many variables as possible should be standardized.

Diffusant Concentration

The rate of diffusion is directly proportional to the concentration of drug in solution at the donor surface of the membrane. The greatest thermodynamic potential of an experimental system would therefore be generated by a saturated solution of the diffusant in the vehicle. The solubility of the diffusant may be increased by incorporating cosolubilizers into the formulation, but these agents may have other influences such as altering the partition coefficient between vehicle and skin, or changing the barrier potential of the membrane. The ease of solubilization of solid drug in suspension-type vehicles is also important if the donor concentration is to be maintained at the surface of the membrane. Furthermore, the viscosity of the formulation may hinder intravehicle drug diffusion and result in a steadily decreasing concentration at the membrane interface. In general, any physicochemical factor that may change the concentration of the drug at the membrane surface will alter the permeation behaviour.

Partition Coefficient

The influence of the donor vehicle composition on the partition coefficient is important in that any change in the partition coefficient will change the drug concentration established in the superficial lamina of the

membrane. It is theoretically possible to formulate a vehicle that has such high affinity for the drug that minimal partitioning occurs into the membrane. Conversely, the permeant may have little affinity for the vehicle and exhibit relatively high partitioning into the membrane. The magnitude of the partition coefficient will be influenced by any factors that affect the potential of the molecule to escape from the donor vehicle, including the degree of ionization, complexation or adsorption.

Equally, the solubility of the drug in the membrane would influence its affinity for that medium. This introduces the drug structure-activity relationship which, generally, suggests that the more lipophilic molecules would have greater affinity for the skin and would partition to a greater extent than polar moieties, however some bipolar character is essential *(12)*. Furthermore, surfactant molecules in the donor vehicle may enhance partitioning by reducing the surface tension between the vehicle and the membrane surface, but may also influence the barrier potential of the membrane *(13)*. Octanol-water partition coefficients have classically been used as the measure of the tendency for a chemical to partition into the lipophilic environment of the skin. However, the usefulness of this parameter has been surpassed by the partition coefficient between water and other organic solvents, for example isopropyl myristate, that simulate the biochemical composition of the skin more closely *(14)*.

Skin Damage

Any pathological or physical condition that alters the character of the stratum corneum will probably alter its resistance to permeation. Diseased skin is generally considered to be more permeable than healthy tissue and several studies have indicated the increased permeability exhibited by skin intentionally damaged by removal of the stratum corneum using adhesive tape, or abraded in some manner *(15,16)*. Although *in vitro* permeation studies would normally use healthy, undamaged skin, there is a possibility that these membranes may be damaged by preparative techniques or by the experimental methodology. Chemicals used to separate skin strata, or organic solvents contacting the media, may denature the lipoprotein environment of the stratum corneum, resulting in more facile permeation *(17)*.

There are conflicting reports in the literature concerning the effect of organic solvents on the barrier potential of animal skin. Some reports suggest that these solvents increase the permeability of the stratum corneum, probably by destructively modifying its dense, keratinized structure *(1,18-21)*. Blank and Scheuplein *(22)* have reported that a mixture of chloroform and methanol

extracts the lipid fraction of the horny layer, forming artificial shunts through the membrane. Onken and Moyer *(23)* have reported that hexane, acetone and alcohol increase the permeability of the skin to water. Conversely, other reports *(24-27)* indicate that organic solvents have little or no effect on the barrier properties of the skin. Prudent experimental methodology would, therefore, avoid contact of the membranes with any chemical that may possibly alter their biochemical composition. The problem is not as important where synthetic media are used as these materials are relatively inert to the commonly employed laboratory chemicals.

Skin Hydration

The stratum corneum is able to absorb large quantities of water, causing swelling of the tissue and an increase in its suppleness *(28)*. However, with the greater moisture content there is also an increase in the permeability of the membrane. Generally the passage of diffusants, including corticosteroids, is much more facile through the hydrated environment of the horny layer than through the compact, desiccated tissue *(18,29,30)*. This has led to intensive investigation into the factors that may promote or maintain hydration of the skin so that drug absorption may be maximized. In this regard urea has been shown to assist in the skin hydration process. This chemical also has keratolytic properties which suggests that its incorporation into topical products should be beneficial in augmenting drug absorption *(31,32)*.

In vitro, hydration of excised skin may be induced by the experimental methodology adopted. Application of occlusive topical formulations, such as lipophilic ointments, form a relatively impervious covering over the skin, preventing the evaporation of transpirational moisture. In steady-state permeation experiments both surfaces of the membrane are usually bathed with the donor and receptor fluids, normally aqueous, and these hydrate the membrane fully within a short period *(33,34)*. The permeation through these membranes therefore resembles *in vivo* diffusion through fully occluded, nonphysiological skin.

Conversely, hygroscopic constituents of topical formulations, such as propylene glycol or glycerol, may absorb water from the skin thereby reducing its state of hydration and subsequent ease of drug passage. Therefore, the state of hydration of biological membranes is an important aspect to be borne in mind when designing *in vitro* diffusion cell methodology. Obviously, the more physiologically the tissues are maintained the greater is the likelihood that correlation will be achieved between *in vitro* and *in vivo* results.

Penetration Enhancers

Certain chemicals have the ability to reversibly alter the resistivity of the stratum corneum to diffusion. The action of these penetration enhancers is distinct from that of organic solvents, for example, in that the latter agents may delipidize the horny layer, irreversibly damaging the tissue. Penetration enhancers induce a transient decrease in the barrier which reforms on the clearance of the agents from the horny layer. The exact mode of action of the penetration enhancers is seldom fully understood. In many cases their presence in the barrier layer affects a number of physicochemical processes simultaneously. The postulation that the enhancers somehow "carry" the drug molecules through the stratum corneum is unlikely. More feasible is the suggestion that the skin environment is modified slightly so that subsequent drug passage is facilitated. Interaction between the enhancer and the keratin fibrils, or enhancement of barrier hydration are two possibilities. The rate of percutaneous absorption of the enhancer and its rate of clearance from the skin would therefore influence its effect on the barrier. Equally possible is the suggestion that many enhancers alter the thermodynamic potential of the drug in the applied vehicle, increasing the ease with which the drug may leave the formulation and enter the skin. Any combination of the mechanisms described above, including damage to the tissue, may be operative simultaneously.

Urea has already been mentioned as a possible penetration enhancer. Other agents that have been investigated or put into clinical practise include dimethyl sulphoxide, dimethylacetamide, dimethylformamide, azone, propylene glycol and numerous pyrrolidone derivatives *(35-38)*. Each agent has characteristic effects on the skin and vehicle. Dimethyl sulphoxide, for example, facilitates the penetration of low, but not of high molecular weight substances. Propylene glycol may beneficially reduce the resistivity of the stratum corneum but may equally increase the affinity of the drug for the vehicle, with little nett result on the overall flux rate. Complicating the experimental situation, the results observed *in vivo* using penetration enhancers do not always parallel *in vitro* results *(39)*.

There are, therefore, numerous factors that may affect the observed permeation rate of a topically applied drug both *in vivo* and *in vitro*, and many factors may influence more than one aspect of the permeation process. It is the complex interaction between the biochemical constituents of the skin, the drug and the constituents of the donor vehicle that may either enhance the permeation of the drug through the membrane, or retain it within the vehicle environment. No mention has been made of other physical stimuli that may increase percutaneous absorption such as the use of ultrasound *(40)* or

iontophoresis *(41)* as these are unique investigations in themselves. The laboratory control of all variables in the percutaneous absorption process is impossible, however as many as possible should be standardized in any experimentation so that valid conclusions may be drawn from the results.

1.4 DIFFUSION BOUNDARY LAYER FORMATION

The importance of maintaining sink diffusion conditions has been emphasized in the preceding discussions of the physical and kinetic events of the permeation process. The drug concentration gradient is the driving force for diffusion and any increase in the drug concentration on the receptor side of the barrier would decrease the gradient magnitude and retard further permeation. In many cases of *in vitro* diffusion cell use, absolute sink conditions do not prevail for the entire experimental period because of accumulation of the permeant in the receptor phase. However, if this increase in concentration is not substantial, if it does not exceed 10% of the donor drug concentration for example, then it may be assumed that sink conditions have not been violated. On the other hand, in certain diffusion cell designs it is possible that the measured receptor chamber permeant concentration would not increase markedly but that the diffusion process would still be retarded because of inferior fluid mixing hydrodynamics and the resultant formation of extensive boundary layers.

The characteristics of the mass transfer within the receptor chamber of the diffusion cell are determined by the nature of the liquid motion: either laminar or turbulent flow may exist dependent on the degree of fluid agitation. Laminar flow is uniform in character with little variation in the velocity of the fluid eddies throughout the chamber. Turbulent flow is irregular in direction and velocity, and mass transfer in these cases is by irregular fluid pulsations. In both cases, the properties of fluid flow at the membrane surface are important as the diffusant molecules that partition into the receptor fluid from the distal surface of the membrane must be rapidly swept away by the bulk fluid motion so that the concentration differential may be maintained across the barrier.

In both flow conditions the velocity of the fluid will decrease in the layers adjacent to the membrane, and will be zero at the barrier surface. This layer of fluid near the membrane that is not agitated at the same rate as the bulk fluid is termed the boundary diffusion layer and is generated by the

viscous, frictional drag forces between one theoretical fluid layer passing over another. The rate of agitation is one of the primary determinants of boundary layer thickness. The greater the degree of agitation of the bulk fluid the greater will be the shearing stress on the fluid layers adjacent to the membrane, and a thinner boundary layer will result. Conversely, slow stirring of the chamber fluid will generate much thicker stationary diffusion layers with a more gradual decrease in the fluid velocity. Turbulent flow is generally regarded as more efficient than laminar flow in the dispersion of these relatively stationary fluid layers. The viscosity of the receptor fluid is equally important in determining the thickness of the diffusion layer. Agitation that is adequate for one fluid may be inadequate for more viscous solvents.

The thickness of these diffusion boundary layers is important in the design of *in vitro* diffusion cells as they present additional resistance, in series, to the mass transfer of the permeant from the donor vehicle to the receptor fluid. This additional resistance may mistakenly be attributed to the membrane medium. Moreover, in certain circumstances the rate-controlling step in the permeation process may become the drug diffusion through these stagnant layers. Figure 2 is a schematic representation of a diffusion boundary layer adjacent to a membrane under conditions of nonoptimum agitation.

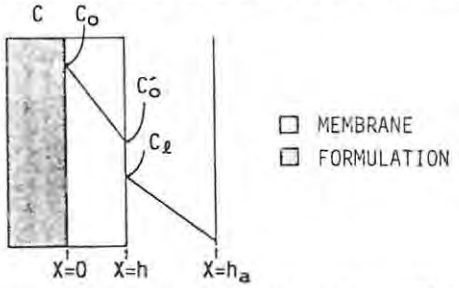


FIGURE 2: Hypothetical diagram of boundary diffusion layer.

The concentration of drug in the donor vehicle (shaded region) is C, which is in contact with the membrane at x = 0. The drug concentration in the first lamina of the membrane is C_o and decreases linearly through the membrane (at steady-state) to the receptor surface (x = h) where the concentration is C_o'. Due to the membrane-receptor fluid partition coefficient the concentration of drug in the fluid layer in contact with the membrane is C_ℓ and, under sink conditions, this decreases to zero at $x = h_a$. This distance marks the end of the unstirred boundary layer and the beginning of the bulk, stirred fluid. Under conditions of ideal, optimized stirring the thickness of the boundary layer (h_a) would be negligibly small and the concentration C_o' would rapidly reduce to zero at a distance (h + dh), where $dh \ll h_a$.

Only under conditions of optimal fluid agitation would the diffusion boundary

layers in the receptor chamber of the diffusion cell be minimized, and, therefore, negligibly influence the overall resistance to mass transfer. A review of the literature indicates that in many instances the diffusion cells employed in experimentation have not been validated with respect to the degree of agitation used. Furthermore, in many cases it appears that the shape of the cell used would not support adequate hydrodynamic fluid transfer to the surface of the membrane (a review of these cells is given in Section 2). In these cases it is assumed that the permeation results obtained must be ascribed to the additive resistivities of the membrane and stagnant diffusion layer, with no indication of the relative magnitude of each.

Optimal cell design and validation is therefore imperative. In cases where their existence has been addressed, the problem of boundary diffusion layers has been overcome in a number of ways. Chien and coworkers *(42)* have applied the theoretical concepts of the Similitude theory initially proposed by Frank-Kamenetskii *(43)*. This theory proposes that if fluid hydrodynamics and mass transfer can be measured under nonideal mixing conditions, theoretical equations may be applied to yield the equivalent mass transfer magnitude that would be achieved under optimal stirring. These authors have therefore used a nonoptimized cell design, but one that has been calibrated, and have applied the necessary equational manipulations to their results.

Stehle and Higuchi *(44)* have assessed the thickness of the boundary layer by monitoring the rate of partitioning of a solute from the aqueous chamber fluid into a lipid membrane. The magnitude of the layer may be estimated graphically from the gradient of a log (sample concentration / initial concentration) *versus* time plot.

The most practically useful method appears to be the monitoring of several steady-state permeation experiments using the *in vitro* diffusion cell and the same permeant, but altering the degree of agitation of the receptor chamber fluid in each case. In these experiments the measured flux rate should increase as the degree of agitation increases. Extrapolation of the flux *versus* reciprocal stirring speed plot to the axis where reciprocal stirring speed is zero will yield an estimation of the flux rate at infinite stirring speed for that diffusion cell, where there are, theoretically, no boundary layers in existence.

Stehle and Higuchi *(44,45)* have used these steady-state measurements to calculate absolute values for the thicknesses of the boundary layers. Other authors *(40,46,47)* have used a modified form of Equation 7 to account for the two additional resistance strata in series (one on either side of the membrane in steady-state experiments):

$$\frac{dM}{dT} = \frac{C}{\frac{2h_a}{D_\ell} + \frac{h}{KD}}$$

Equation 12

where h_a is the thickness of the diffusion boundary layer and D_ℓ is the diffusion coefficient of the permeant in the liquid phase. Using this equation the thickness of the boundary layer at each stirring speed may be estimated, however the diffusion coefficient of the permeant in the liquid must be known.

In practice it is not essential to calculate these thicknesses if the results of the diffusion cell validation experiments show that optimal agitation is being applied to the system. If this is the case then increasing the stirring speed over a certain value will not increase the measured flux rate any further and it may be assumed that the boundary layer thickness has been minimized for that particular cell configuration and stirring system. Here the contribution of the boundary layer to the overall diffusive resistance may be considered negligible and a more accurate estimation of the resistivity of the membrane would result. Therefore, an important facet of the *in vitro* experimentation is that some validation of the diffusion cells with regard to optimized agitation is carried out.

2. *IN VITRO* DIFFUSION CELL DESIGN

2.1 BASIC PRINCIPLES OF DESIGN

A multitude of experimental methods have been developed to investigate the percutaneous delivery of topically applied drugs in an attempt to isolate the physical and chemical factors that govern drug absorption. In many instances the diverse experimental techniques tend to obscure absorption controlling factors and complicate interstudy comparisons rather than clarifying the immensely complex transdermal absorption process. Moreover, lack of agreement between findings has often been a result of shortcomings in the *in vitro* methodology. The objective of this research is to find some correlation between laboratory results and the transdermal absorption experienced by living subjects so that the necessity for *in vivo* experimentation may be curtailed.

The benefits of utilizing an *in vitro* cell system for the preliminary testing of drug diffusion in the laboratory are obvious. The environment, specific permeation parameters and variables may be controlled in an attempt to elucidate specific factors affecting the kinetic processes, prior to undertaking studies in human volunteers. The permutations of these variables that are selected for study will obviously dictate the design and protocol of the research. Experimental design that allows the greatest number of variables and different variable permutations to be investigated by the same *in vitro* diffusion apparatus is highly desirable. In this manner a single, versatile diffusion cell may be able to yield results which help to elucidate the kinetic processes involved in more than one of the permeation steps. By reviewing the experimental systems that have been reported, the beneficial features of many designs may be incorporated into one composite which should then provide optimal methodology for the specific project at hand. Drug absorption factors which may require consideration when selecting an *in vitro* system include:

1. The intrinsic diffusivity of the permeating molecule, its structure-activity relationship and, thereby, its apparent diffusion coefficient.
2. The route of penetration that will predominate during the experiment and the relative extents of drug binding and metabolism that will occur.
3. The rate-limiting factor in the permeation process: drug solubilization and/or diffusion in the formulation, partitioning from the vehicle, diffusion through the test membrane or partitioning and removal by the

receptor phase.

4. The intrinsic barrier potential of the test membrane and the effects that vehicle components may have on its retardive properties. Hydration of the membrane may be important in this regard as may be the presence of penetration enhancers. Interspecimen variability between membranes of the same type may greatly influence experimental results.

A diffusion cell system cannot duplicate exactly the events that occur in living human tissue, the clearance of diffusant by the vasculature and enzymatic metabolism are especially difficult to simulate *in vitro*, as are the pharmacodynamic events such as corticosteroid induced vasoconstriction. However, as long as diffusant clearance is not the rate limiting step to diffusion and metabolism is not extensive, it should be possible to correlate *in vitro* diffusion results with *in vivo* data *(48-50)*.

DIFFUSION CELLS WITHOUT MEMBRANES

A number of diffusion systems have been evaluated that have no membranous barrier to the passage of permeant *(14,51-61)*. Typically the topical formulation is contained within a vessel and is immersed into an immiscible, agitated receptor liquid that is thermostatically maintained at constant temperature. The appearance of drug in the receptor phase is then analytically monitored. These systems have generally been abandoned because of lacking similarity to *in vivo* diffusion or even to diffusion through barrier systems *in vitro*. At most they can be described as drug partitioning systems between two or more immiscible phases in contact and they may provide some information concerning the release of drug from the donor vehicle. No parallel is implied between this release and that occurring when the vehicle is applied to the skin, although this may exist.

DIFFUSION CELLS WITHOUT RATE-LIMITING MEMBRANES

These techniques mainly investigate interactions between drug and topical vehicle, and the release characteristics from the formulation. The donor vehicle may be contained within an open glass jar or Petri dish covered by cellulose medium or similar porous membrane that will prevent dispersion of the formulation throughout the receptor phase but will in no way influence the movement of drug molecules into the liquid. The container is typically immersed into an immiscible, agitated, thermostatically controlled receptor solvent and the increase in concentration of diffusant in the liquid receptor

phase is then analytically monitored as a function of time. The mass of drug released from the formulation is proportional to the square-root-of-time, provided the membrane is in no way rate limiting and less than 60% of the total amount of drug present in the vehicle has been released *(31,62,63)*. The rate of drug release, therefore, decreases exponentially with time and the absence of a lag time in the permeation profile would indicate that there are no important diffusion controlling steps mediated by the separating membrane. Release characteristics in these cases are governed almost entirely by the donor formulation. These tests may be valuable in elucidating occurrences such as drug particle solubilization within the formulation and the potential of the molecules to partition into the receptor solvent, but they have limited relevance to the complex process of percutaneous permeation.

Modified ointment jars, with membranes (usually cellulose media) secured over the jar mouths, have been used extensively as donor formulation containers in this field of research *(32,64-68)*. The membrane is nondiscriminatory in its barrier properties and bidirectional diffusion of the components of both chambers is usually experienced. Formulation constituents such as polyethylene glycol fractions have been detected in the receptor fluid while the movement of water into the donor chamber, and its interaction with formulation ingredients on the donor side of the membrane, has resulted in the precipitation of the drug, due to low water solubility, or dissolution of the donor vehicle *(69,70)*.

While several liquids have been used as receptor media in these studies, it has become apparent that aqueous systems may be nonideal for relatively water insoluble drugs. From their studies Turakka *et al. (64,71)* conclude that the water content of the donor vehicle appears to be the most important factor for *in vitro* hydrocortisone release into propylene glycol receptor phase. They found that the corticosteroid was not released into their in vitro system from anhydrous dosage forms. Furthermore, they report the release of the acetate ester of hydrocortisone to be less than that of the parent alcohol, in direct contradiction to *in vivo* observations and *in vitro* studies using biological membranes.

Several variations have been presented by Busse *et al. (51)* and Malone *et al. (72)* using filter paper soaked in isopropyl myristate as the receptor phase, and Mazzo *et al. (73)* have modified tablet dissolution apparatus to test transdermal therapeutic systems.

Although these methods are useful as screening tools for drug release or for detecting drug-vehicle interactions, their limitations due to intimate

contact of donor and receptor vehicles and the aselectivity of the membrane makes extrapolation of results to *in vivo* usage unreliable. The limitations of the methodology are also exemplified by the fact that nearly all of the published research concerns the release of molecules from viscous, lipophilic, ointment bases which are essentially immiscible with aqueous receptor phases. Little work has been attempted using aqueous lotion, cream or gel formulations. Hadgraft *(11)* has warned that spurious results may be obtained using this methodology if aqueous formulations are tested due to water diffusion into the dosage form. It is therefore accepted that a versatile *in vitro* diffusion system would require a rate-limiting barrier medium to separate the donor formulation and receptor phase so that many of these shortcomings described above may be obviated.

DIFFUSION CELLS WITH RATE-LIMITING MEMBRANES

Several diffusion cell designs incorporating rate-limiting membranes have been reported in the literature, each design having specific features which it is hoped will overcome certain experimental limitations. Most designs share common features: two chambers, one containing the donor vehicle and the other containing agitated receptor phase fluid, the two chambers being separated by the membrane under test. The appearance of drug in the receptor phase (or diminution from donor phase) may then be analytically monitored as a function of time. The donor vehicle is assumed to expose the membrane to a constant concentration of the drug and the receptor phase is assumed to maintain sink clearance conditions for the permeant. Barry *(2)* has reported that a depletion of donor phase or an increase in receptor phase concentration not exceeding 10% does not significantly violate zero-order flux conditions or deviate from the initial thermodynamic driving force for diffusion.

The influence of adequate receptor solvent agitation on the results obtained from *in vitro* diffusion experiments has been shown by Lovering and Black *(74)*, and by other workers *(44)*. They observed that as stirring rate increased, the thickness of the unstirred boundary diffusion layer decreased and drug permeation rate increased. Moreover, if stirring increased sufficiently the rate of detected permeation would no longer be partially governed by the diffusion layer thickness. It is noted from the literature that many experimental observations are made when boundary diffusion layers represent a significant portion of the total barrier presented to the diffusing solute. Obviously the greater the portion of this representation the less accurate are the results obtained with respect to the membrane itself and the greater will be the deviation between *in vitro* and *in vivo* results.

In some cases the existence of the diffusion boundary layer is acknowledged but is ignored in comparative work because of its uniformity throughout the experiments *(75)*, or assumed to be negligible *(76)*. This is acceptable as long as it is borne in mind that calculation of the diffusion coefficient will include the contribution of the boundary layer barrier and thereby only yield an apparent diffusion coefficient value. In other cases failure to calibrate the diffusion cells with respect to diffusion layer thickness may lead to dubious results *(1,77-79)*.

Although several solvents have been used for the donor and receptor vehicles, the vast majority of the reported experiments use aqueous media because the assay of drugs in aqueous solution is facile by a number of techniques. However, drug solubility must be considered when aqueous solvents are used. If the diffusant is only slightly soluble in the receptor fluid then it will not partition from the distal surface of the membrane and further diffusion will be retarded. Problems of this nature have been reported for oestradiol and testosterone *(80,81)* and other compounds *(49)*. This has been cited by Bronaugh and Stewart *(82)* as a reason for the misleading results observed in some standard diffusion cell techniques, and for discrepancies between *in vivo* and *in vitro* results. These authors caution that compounds having an aqueous solubility approaching 10 mg l^{-1} or less would certainly demonstrate limited *in vitro* partitioning into hydrous receptor environments. Wester and Maibach *(83)* report that flow-through receptor cells are more beneficial in this regard than static type cells because sink conditions can be maintained more adequately in the former for sparingly soluble diffusants as saturation is avoided. Diffusants that may not partition into aqueous receptor phases *in vitro* may be sufficiently soluble in biological fluids in vivo to maintain adequate clearance and, thereby, sink diffusion conditions. Permeant solubility is a complex problem assumed to be common to a number of experiments and is especially apparent when investigation involves corticosteroids which are inherently insoluble in water.

In addition, the susceptibility of aqueous media to microbial contamination, when considered with its potential for the aqueous degradation of diffusant molecules, makes the choice of a nonaqueous solvent for donor and receptor vehicles fairly appropriate. The question of hydration should also be addressed when membranes are immersed for prolonged periods in aqueous vehicles. From published literature the effect that hydration may have on permeation appears to vary dependent on the drug under investigation and membrane characteristics *(84-86)*. On the other hand, the use of alcoholic or nonpolar organic solvents must in no way affect the biochemical composition of

the barrier membrane. Although Sloan *et al. (84)* report that a three minute wash of hairless mouse skin with methanol had only a marginal effect on the flux of theophylline, Bronaugh and Stewart *(82)* observed that methanolic and ethanolic receptor solvents damaged full thickness rat skin *in vitro* as assessed by the increases in flux rates of cortisone. However, these researchers motion that simply using a receptor solvent which will solubilize the diffusant and not damage the membrane is inadequate, they maintain that the establishment of greater overall lipid solubility in the receptor is necessary to compete with the lipophilic properties of the membrane.

Bearing these points in mind, a nonviscous, lipophilic fluid such as isopropyl myristate appears to be an ideal choice for the receptor medium *(87)*. It is a useful solvent for the majority of molecule types employed in transdermal diffusion studies and its lipid nature will not support hydrolysis-mediated molecular rearrangement or cleavage reactions. In addition, isopropyl myristate is largely favoured because of its bipolar character that tends to simulate the heterogeneous environment of the skin *(14)*. Equally, the possible deleterious effects of isopropyl myristate on the barrier properties of the skin must not be ignored. As the lipophilicity of the receptor phase increases the possibility for extraction of endogenous lipid components from the skin tissues increases *(82)*. It has been proposed that isopropyl myristate pretreatment of hairless mouse skin increases its permeability to certain diffusants *(84,88)*. Notwithstanding these observations isopropyl myristate appears to have little deleterious effect on the composition of biological media while presenting a highly favourable medium of biphasic nature for permeant partitioning.

Cells for Determining Steady-State Permeation

These cell designs usually comprise two similar chambers containing permeant in solution and receptor solvent respectively *(77,89-93)*. They are useful for assessing the intrinsic diffusivity of a molecule through the membrane under test, or the effect of partition coefficient, pH or boundary layers on diffusion in the absence of any parameters (such as formulation composition) that may enhance permeation.

In many instances the method of fluid stirring has dictated the variations in cell design. An early bichamber design *(94)* rotated about an axis parallel to the plane of the membrane forcing an air bubble in each chamber to flow through the fluid contents. Julian and Zentner *(40)* have used an advanced design of the immersed ointment jar incorporating two magnetic stirrer bars, one in the bulk receptor solution and one resting on the donor surface of the

horizontal membrane.

A more sophisticated donor chamber is described by Bottari and coworkers who used a recessed stainless steel *(95)* or polymethyl methacrylate *(76)* plate to contain the formulation, covered by hydrated silicone membrane, and the assembled cell was immersed into a beaker of the stirred receptor medium. Commendably, the authors ensured that sink diffusion conditions were maintained throughout the experiment. A similar cell has been used by Broberg *et al. (83)*.

Comparatively large volume, large diffusion area cells have been moulded from perspex blocks *(13,96,97)* or lucite *(98)*, see Figure 3a. The cell halves securing the membrane were clamped together by nuts and bolts and agitation was generated by bar magnets or immersible stirring units. Stoppered sampling ports allowed aliquots of receptor solution to be removed at suitable time intervals. Although facilitating drug determination in the receptor phase, these large diffusion area cells are only useful for tests employing membranes that are in copious supply.

Several researchers have investigated the benefits of an inverted T-shaped cell with the membrane covering both horizontal ends, or have used identical L-shaped chambers clamped together to resemble a U-tube *(99,100)*. Adequate mixing of the fluid in the arms of the cell would appear to pose a problem in this design. Recycling of the fluid from a reservoir or bubbling an inert gas through the fluid are methods of agitation that have been practised *(101)*, however variability in the results from repetitive experiments is generally high with this design. Lovering and coworkers *(102)* have modified the cell to contain an internal means of agitation and have attached the silicone membranes to the cell ends with adhesive. Although fluid mixing may be improved, the authors make assumptions concerning boundary layer formation without adequate calibration data for the cell system.

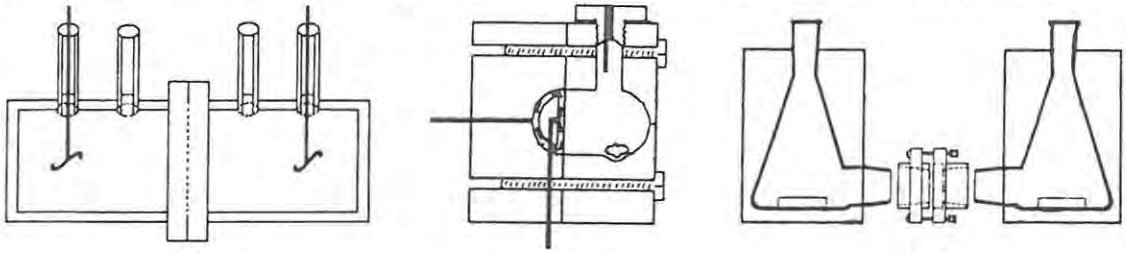


FIGURE 3: *In vitro* diffusion cell designs from references 98, 35 and 44, respectively.

Flow-through cells have been used effectively in which the contents of both donor and receptor cells are continuously recycled via a pumping mechanism *(7)*, or a combination of magnetically stirred donor chamber and flow-through receptor chamber has been used by Astley and Levine *(35)*, see

Figure 3b. In many cases the fluid inlet is directed onto the surface of the membrane which may generate some degree of strain. Flow rates cannot be too great and, thus, it is doubtful that regions of the cell distant from the inlet or outlet apertures are thoroughly mixed by the fluid circulation. This technique obviously requires a large reservoir of receptor fluid with which the membrane may be bathed over the course of the experiment and, therefore, the method of analysis for the permeant must be extremely sensitive to assay these dilute concentrations.

The majority of the steady-state cells reported in the literature consist of two chambers, usually constructed of glass, separated by a membrane-fastening device *(103)*. These chambers may simply be two Erlenmeyer flasks that have been modified to support a membrane between connecting ports on each vessel *(104,105)*. The flasks may be independently agitated via magnetic stirrer and immersed in a constant temperature water bath. If the flasks are independently heated by water jacket *(44)*, the membrane holder is exposed to ambient conditions and this may generate a temperature gradient between the bulk cell contents and the fluid juxtaposed to the membrane, see Figure 3c. The fluid mixing efficiency at the membrane interface appears to be questionable in most of these modified flask designs. A similar cell design, with inferior hydrodynamics, is that proposed by Wurster *et al. (106)* which has no internal means of agitation but is shaken gently in the plane of the membrane, see Figure 4a. In this design a cylindrical constriction connects the membrane with the bulk chamber fluid and boundary diffusion layers are, therefore, assumed to be extensive. The replacement of one chamber with a specially designed block aperture allowed the application of gel or gas to the donor side of the membrane.

Durrheim and coworkers *(107)* have used a simple bichamber glass or polycarbonate cell design, with good diffusion area to volume ratio, in the form of two cylindrical half-cells. Each chamber had two vertical ports: one for sampling the receptor solution and the other to accommodate the shaft of the motorized stirrer. The propellers attached to these shafts were positioned close to the membrane surface which generated gentle but adequate agitation.

Harper Bellantone and colleagues *(41)* have also used a cell consisting of two cylindrical chambers with mixing generated by teflon bar magnets, see Figure 4b. The constriction of the chamber diameter near the membrane surface appears to have posed some problem with air bubble formation and, presumably, would have hindered fluid mixing.

Touitou and Abed *(108-110)* have employed a shaking technique to agitate their cylindrical cells, instead of using an internal means of agitation.

Although the authors state that no stagnant diffusion layers were detected, they do not elaborate on the methodology used to validate this statement. It does not seem conceivable that, in the absence of a void volume or large air bubbles within the cylindrical cells, horizontal shaking of the closed system would induce agitation of any significance.

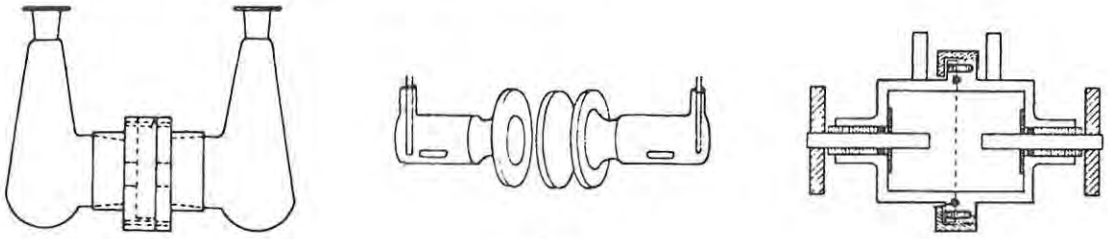
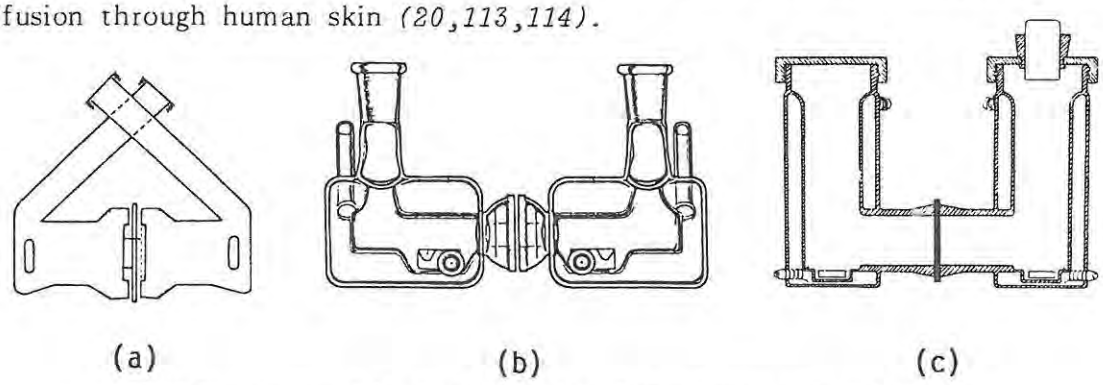


FIGURE 4: *In vitro* diffusion cell designs from references 106, 41 and 111, respectively.

A complex, dual chamber, machined brass diffusion cell has been evaluated by Flynn and Smith *(111)*, see Figure 4c. Agitation was generated in each chamber by relatively large, specially formed, teflon stirrers which were mounted in the same vertical plane as the membrane. This necessitated horizontal stirrer shafts to protrude from each chamber and gasket seals to prevent any leakage of chamber fluid through the shaft housing sleeves. A synchronous motor was used to drive the stirrers in each chamber at the same rate. This design appears to be ideal in many respects: good membrane area to volume ratio (0.67), total temperature control by complete immersion of the assembled unit, uniform sealing pressure on the membrane and, most importantly, optimal agitation at the surface of the membrane. However, the complexity of this design dictates that it be manufactured by an engineering concern and will require specific mountings for motors, shafts and cogs. A similar means of agitation has been used by Galey *et al. (112)* in their glass cell.

The most useful design in this regard appears to be one proposed by Barry and coworkers *(36,85)*, see Figure 5a. Two glass chambers with a relatively small diffusion area, more in keeping with the size of readily available biological membranes, are immersed horizontally in a water bath. Agitation is effected by teflon-coated bar magnetic stirrers and stainless steel mesh has been used to support delicate membranes. Sampling is undertaken via inclined ports which are closed by Parafilm or glass tubes. There is no possibility that air bubbles may become trapped under the membrane and there is no hydrostatic fluid pressure that may distend the barrier. It appears that the bar magnets in each chamber are stirred from both ends of the cell rather than from below, complicating the mechanics of the system. It is proposed that a single stirrer positioned below the chambers, provided they were not

cylindrical in shape, would effectively induce synchronous stirring of the bar in each cell. In addition, the connection of the inclined sampling port distally to the membrane flange may not support efficient mixing of the fluid in this tube. A modification of this design has been used to measure vapour diffusion through human skin *(20,113,114)*.



(a) (b) (c)

FIGURE 5: *In vitro* diffusion cell designs from references 85, 117 and 118, respectively.

Probably the most comprehensive investigation into the hydrodynamics of *in vitro* diffusion cells has been carried out by Chien and coworkers at Rutgers University. They have developed and validated a number of cell designs using highly complex theoretical models initially proposed by Frank-Kamenetski *(43)*. In most cases they have adopted nonideal cell designs with inferior mixing potential and have mathematically idealized their results using theoretical dimensionless parameters such as the Sherwood, Schmidt and Reynolds numbers.

A sophisticated bichamber design is that reported by Valia and Chien *(81,115,116)* and later characterized by Tojo *et al. (117)*, see Figure 5b. Each half of the cell comprises a horizontal, jacketed cylinder with a vertical, stoppered, sampling port and a unjacketed membrane connecting flange. Each chamber has a depression in the cylindrical section to accommodate a star-head magnetic stirrer. It is assumed that fluid mixing into the constriction juxtaposed to the membrane would not be ideal, especially when one considers the difficulty in achieving efficient agitation in a horizontally orientated cylinder. Additionally, it is reported that exposure of the flange to ambient conditions generates a small temperature gradient between cell bulk and membrane surface. After introduction of a drug aliquot homogeneity of the chamber solution ensues after approximately one minute of stirring. This value compares very favourably to the 10 minutes required for equilibration in the Franz cell (described below). Furthermore, the calculated theoretical diffusion boundary layer thickness was five times smaller for the Valia-Chien cell than for the Franz design.

A similar steady-state diffusion apparatus (Figure 5c) has been developed and evaluated by Chien and coworkers *(118-122)* and has subsequently been used

by several researchers *(123-126)*. The vertical arms of each L-shaped chamber are water jacketed for temperature control, each has a sampling port with cap to prevent evaporation of the fluid, and each has a recessed, stirring platform to accommodate a teflon stirrer bar. Stirring of the fluid in the horizontal arms of the chambers may not be ideal and the absence of temperature control along these arms may generate gradients between the membrane surface and bulk fluid. As with the previous design, cell shape here deviates markedly from the ideal and the authors propose that this may validly be counteracted by the application of correction factors based on a theoretical mathematical hydrodynamic model.

These steady-state diffusion cells are useful in that a number of kinetic permeation events may be monitored simultaneously. The mass of drug entering the membrane may be assessed by monitoring its diminution in the donor solution and the fraction of drug bound within the membrane may be assessed by the difference in mass entering the membrane and that partitioning into the receptor chamber *(30)*. Moreover, the cells are extremely useful for determining intrinsic diffusivity of a molecule through a specific membrane in the controlled absence of factors that may influence the permeation process.

Cells for Simulating *In Vivo* Conditions

An *in vitro* permeation system that parallels *in vivo* absorption more closely would require a hydration and temperature gradient to be established across the membrane, with only the receptor surface of the medium being exposed to the solvent *(127)*. In this situation the formulation interacts with the skin and atmosphere and permeation may thus be studied under experimental conditions most applicable to *in vivo* use of the formulation. In this experimentation the characteristics of the donor vehicle may vary widely from the true solutions used in steady-state experiments. A film of drug deposited by solvent evaporation, one of the numerous types of topical formulations, or even a transdermal delivery device, may be used as the drug source. Other benefits include the ability to control parameters of the donor compartment such as humidity, thereby simulating occluded or unoccluded clinical conditions. Additionally, the membrane may sequentially be treated with chemicals before or during exposure to the permeant to acertain what effect they have on the retardative properties of barrier medium *(2,127-130)*. The distinct advantages of these *in vivo*-mimic systems are apparent in comparison to the steady-state apparatus described previously.

In most investigations it is assumed that the drug concentration in the donor phase does not diminish significantly during the time-course of the experiment. After the initial transient period of membrane saturation, and provided sink conditions are maintained, the concentration gradient across the skin and the drug permeation rate should remain constant. This may provide valuable information regarding the steady state diffusion and lag times when semisolid formulations are applied to the membrane.

However, this infinite dose/steady-state situation does not exactly parallel the formulation use *in vivo* where a finite amount of product is usually spread thinly onto the skin. The concentration or solubility of the drug in this applied layer may change as water evaporates from the dosage form or formulation constituents are absorbed by the skin, thereby changing the thermodynamic potential of the diffusant in the vehicle *(131)*. Therefore, if it is necessary to simulate *in vivo* usage as closely as possible, several factors favour a finite donor dose technique as proposed by Foreman *et al.* *(132)* and Franz *(50)*, which has subsequently been used by other workers *(85, 86,127-130,133-135)*. This method requires that a finite volume of drug solution, in volatile solvent, be applied to the membrane and evaporated to dryness to form a film of drug on the diffusion medium. Permeation of drug from this film passes through a maximum value and then declines as the permeant is exhausted from the deposited layer *(136)*. However, while it may be fairly facile to apply an even film of drug onto the membrane by solvent evaporation, it is postulated that spreading a finite amount of viscous ointment, for example, onto fragile human stratum corneum in a thin, even layer would be extremely difficult. In these situations the use of an infinite dose technique is the only feasible method of experimentation.

Most of the glass cells reported in this experimentation are of similar design: a lower, agitated, solvent-filled receptor chamber with an inclined, stoppered, sampling side-arm. The membrane is mounted horizontally between the flanged edges of the lower chamber and the upper donor container, which are held together in some mechanical fashion making the joint water-tight. Fluid is filled into the cell to the level of the membrane, so that no hydrostatic strain is imposed on the medium, and any air bubbles which form may be expelled through the sampling port by tipping the cell. The chamber is usually immersed in a water bath to the level of the flange so that the donor vehicle is not heated. A number of these cells may be arranged around a single magnetic stirrer for replicate runs.

A cell design proposed by Coldman *et al.* *(25)* and subsequently used by several researchers *(53,137-139)* consists of a vertical cylindrical chamber with an inclined sampling side-arm, of slightly smaller diameter, attached to

the cell body near its base, see Figure 6a. Agitation of fluid within the body was generated by a polyethylene sail attached to a teflon bar magnet. The authors report that this provided efficient mixing but do not present any validating data. It is assumed that a large portion of the receptor fluid would be contained within the sampling side-arm and would not mix adequately with the bulk fluid of the cell. Fluid mixing and mass transfer within the constriction connecting the membrane to the bulk cell may also be nonideal.

A very useful design in this regard is that used by Barry and coworkers *(85,86,129,130)*, see Figure 6b. This is a modification of their steady-state diffusion cell described previously, with a modified donor chamber containing an innovative well-like recess which prevents spillage of the donor vehicle when the cell is tipped to expel air bubble. One possible drawback of this design is the connection of the inclined sampling port near the base of the cell which may not support efficient mixing of the fluid in the sampling tube with that in the bulk cell. The connection near the base dictates that the tube be especially long so that its orifice is positioned above the level of the membrane and hence a substantial proportion of fluid is contained within this sampling port.

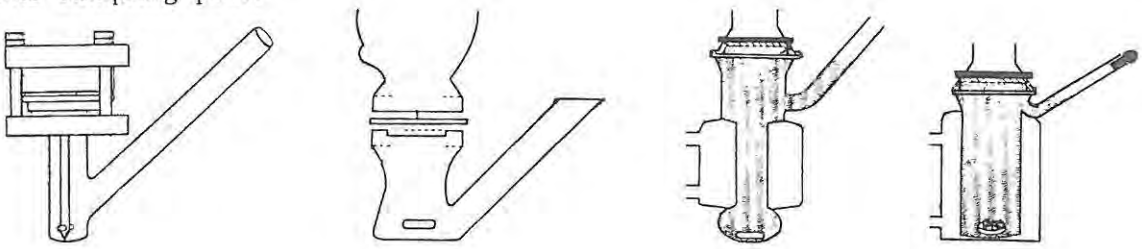


FIGURE 6: *In vitro* diffusion cell designs from references 25, 85, 50 and 42, respectively.

Franz *(50)* has designed a cell system that has subsequently been commercially marketed and used by numerous researchers *(39,88,109,140,141)*. The cell has the normal bichamber arrangement but has a dumbbell-shaped receptor chamber (see Figure 6c). An unstoppered sampling port is connected to the upper segment of this compartment and, surprisingly, only the central, cylindrical, portion of the receptor chamber is surrounded by a thermostated water jacket. Agitation is generated by stirrer bar in the lower, ellipsoid bulb. Ogiso *et al. (142)* have used the Franz cell and they report good correlation with *in vivo* drug absorption studies conducted in parallel. This report exemplifies that even though mixing hydrodynamics may be inadequate, the system may still be useful for screening purposes. Sheth *et al. (37)* have used the Franz cell but have incorporated a specially manufactured elongated stirrer bar, presumably to improve mixing hydrodynamics.

Keshary and Chien *(42,143)* have summarized the shortcomings of the Franz

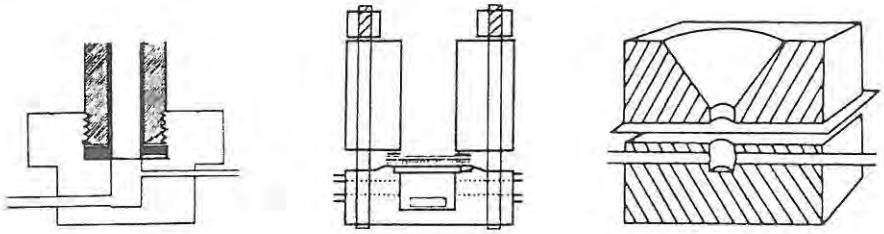
cell after comparative evaluation, and Chien an Valia *(115)* have compared the hydrodynamics of this cell to their own design. They report that the architecture of the Franz cell does not provide adequate solution hydrodynamics, mixing efficiency and temperature control required for quantitative permeation evaluations. Examination of the Franz cell design offers immediate explanation for some of these inadequacies: it is improbable that adequate agitation of the entire receptor cell contents may be transmitted from the stirred lower bulb, through the narrow cylindrical connection to the wider upper compartment. The boundary diffusion layers are reported to be fivefold greater in the Franz design than in the Valia-Chien cell *(115)*, and there is relatively poor vertical permeant and thermal transfer. Receptor chamber sampling would therefore take place from a heterogeneous solution with respect to drug concentration. Obviously these problems are all greatly amplified if solvents more viscous than water are used in the system.

On the basis of these shortcomings Keshary and Chien have proposed a number of modifications to the Franz design (see Figure 6d). Their stoppered receptor compartment is a simple cylinder, shorter in height that the Franz design, completely enclosed by a water jacket and a star-head magnet is used to agitate the fluid. Equilibrium temperature maintenance, boundary diffusion layer thickness and solution mixing efficiency were substantially improved by these modifications. It can therefore be seen that improvements in the hydrodynamic fluid flow characteristics may yield vastly improved penetration profiles of greater validity.

A number of alternative designs for simulating *in vivo* conditions have been reported in the literature. Washitake *et al. (93)* have used a modified T-shaped cell, horizontally shaken, to investigate the diffusion of betamethasone 17-valerate from ointment bases through egg-shell membrane. Cells having a flow-through receptor compartment instead of a sampling port have been used extensively. Sink conditions are relatively easily maintained in flow-through systems as permeant is immediately swept away by the fluid *(144)*. The flow-through receptor chambers are generally much smaller than the static cell type because several cell-volumes of receptor fluid must be pumped through the chamber during the course of the experiment. To maintain the total amount of fluid used at manageable levels the cell size must, therefore, be relatively small. Furthermore, most cells have a relatively small diffusion area making them economical with respect to membrane requirements. However, the formation of air bubbles is reported to be a problem in these systems, even small bubbles trapped beneath the membrane will reduce the diffusion area substantially. The use of in-line bubble traps and the degassing of solvents,

especially hydroalcoholic fluids, usually eliminates this drawback.

The inlet tube of the design used by Foreman and coworkers *(132,135)* directed the fresh solvent against the centre of the membrane undersurface, while effusate was removed via a port at the base of the cell. Obviously, appropriate receptor cell shape would enhance mixing hydrodynamics and it is assumed that basic cylindrical chambers *(145,146)*, see Figure 7a, are not ideal in this regard. Stirrer bars have been included into the flow-through design to improve mixing efficiency *(12,21,147,148)* and in some cases the fluid agitation is totally dependent on the magnetic stirrer and no reliance is placed on the infusion of fluid for agitation *(149)*, see Figure 7b. The hydrodynamics of these systems is therefore assumed to be superior to that of the solitary flow-through arrangement.



(a) (b) (c)

FIGURE 7: *In vitro* diffusion cell designs from references 145, 149 and 26, respectively.

Several modifications of this design have been promulgated in an attempt to relieve the researcher of the repetitive sampling and solvent replacement duties. A continuous flow-through stainless-steel cell connected to an automatic sample fraction collector has effectively been employed by Akhter and coworkers *(26,150)*, see Figure 7c. Several of these cells may be mounted in a circular turntable which will sequentially move the collection vials under the effusion ports of the cells.

A similar, teflon cell design is described by Bronaugh and Stewart *(15,151-153)* although here the inlet tube diameter is approximately equal to the depth of the chamber and, to create a slight back pressure, the chamber has a smaller outlet tube diameter which ensures intimate contact of fluid with the membrane. Again, several of these cells may be fitted into a heated block and sampling effected by fraction collector. The performance of this cell configuration was compared to results from static diffusion cell systems for the permeation of tritiated water, cortisone and benzoic acid through rat skin using different receptor phase solvents. The results demonstrate insignificant difference in the absorption profiles obtained from the two cell designs and good correlation between these data and *in vivo* studies. These results are important from a developmental point of view as they indicate that if the receptor medium is optimized for the specific permeant and membrane

system under test, in conditions of optimal agitation, the flow-through diffusion cell design offers no obvious advantages over the static fluid design with respect to the kinetic data generated.

Therefore, although the flow-through system has several advantages over the static diffusion cells, their design is complex from a manufacturing viewpoint, and therefore expensive, and is totally dependent on the synchronization of pump and fraction collector. Moreover, overt performance superiority of the flow-through system compared to optimized static fluid cells has not been clearly established.

2.2 MEMBRANES FOR USE IN PERMEATION EXPERIMENTS

HUMAN SKIN

It seems obvious that human tissue would be the best medium to use in a diffusion cell so that appropriate extrapolation of conclusions to the *in vivo* situation could occur. Excision of the skin does not alter its permeability properties significantly, provided the stratum corneum remains intact, and several studies have shown that the stratum corneum performs similarly *in vitro* and *in vivo*, even after several postmortal days *(2,17,112,154)*. It should, therefore, be possible to design representative *in vitro* systems using excised human or animal tissue, as a similarity between laboratory animal and human skin has been observed in several studies *(112,147,155)*.

The use of human skin for experimentation is not without problems: its availability is limited and permeability varies greatly between specimens taken from the same or different anatomical sites of the same donor (27% variance *in vivo* and 43% *in vitro*), and greater variations (45% *in vivo* and 66% *in vitro*) are noted between specimens from different subjects or different age groups *(28,85,89,103)*. Complicating these difficulties, metabolism and biotransformation of chemicals applied to the skin may continue for long periods after excision of the tissue from the donor *(116)*, and may be extensive in some cases. Marzulli *et al. (48)* report that residual enzymes in the stratum corneum metabolized permeating testosterone molecules to the extent that only 5% of the applied dose penetrated as the parent compound. This variance necessitates the use of a large number of skin samples to obtain representative, average results.

Full Thickness Skin

Tissue sampling, handling and preparation procedures will vary dependent on the study being undertaken and composition of the membrane required. The most satisfactory source of human tissue is that obtained from cadavers or amputations. This tissue may be stored in the frozen state for prolonged periods without any significant alteration in its barrier potential *(17,35,147, 152,156,157)*. The processing of full thickness skin (stratum corneum, epidermis and dermis) is fairly simple but requires a dermatome. Typically *(2,25,26,129,130)* excess fat is trimmed away from the dermal side of the excised skin and the tissue layer is clamped between metal plates and frozen at approximately -24 °C. The plate in contact with the stratum corneum is warmed slightly so that it may be removed while ensuring that the dermal side of the tissue adheres to the lower plate. A dermatome may then be used to excise a strip of tissue of desired thickness (usually 430 μm) from the thawing surface. Specimens containing a relatively high density of hair follicles may pose a problem in this cutting procedure and should not be selected for full thickness studies. Alternatively, a dermatome may be used to excise strips of tissue of the required thickness directly from the cadaver or amputated limbs *(25,35,135,137,156,158,)*. These strips may then be frozen and stored until required without any further manipulation.

Other sources of full thickness skin that have been used in laboratory diffusion experiments are neonate foreskin *(83)*, callous *(106)*, and scalp tissue *(27)*, however these membranes are atypical in that they have a greater permeability than skin from other anatomical sites, or have been formed in response to pressure abrasion.

If full thickness skin is used it must be borne in mind that *in vivo* the permeating molecule would not have to traverse the dermal layer, the vasculature at the dermo-epidermal junction (at a depth of approximately 200 μm) would rapidly clear any diffusant reaching this interface *(153)*. Conversely, this is an additional resistive pathway to diffusion introduced by *in vitro* methodology. It has been suggested that the presence of the dermis in excised diffusion membranes may present a significant, additional, "artificial" barrier to the diffusion of lipophilic moieties *(82,148,159,160)*.

Stratum Corneum Plus Epidermis

The relatively thick aqueous environment of the dermis may present an unfavourable partitioning environment for lipophilic diffusants and, therefore, *in vitro* experiments are best conducted, for certain diffusants, without the

dermis in place. To overcome these problems, Bronaugh and Stewart *(152)* have simply sectioned samples of skin with a dermatome set at a depth of 200 μm, the approximate thickness of the stratum corneum plus epidermis. This appears to be the easiest method of sample preparation and has the added advantage of not exposing the tissue to possibly deleterious chemicals.

Conversely, several chemical techniques have been investigated for the isolation of the stratum corneum and epidermis from dermis and subdermal tissue. The most facile appears to be the heat separation technique of Kligman and Christophers *(157)*, and modifications thereof *(28,161)*, which requires the skin to be heated to 60 °C for two minutes (by simple immersion in water or clamping between previously warmed metal plates) after which the horny layer/epidermis may be lifted from the dermis using blunt dissection where necessary.

Exposure of the organ to ethylenediaminetetracetic acid solution *(158)* or ammonia fumes *(157)* has a similar splitting effect at the dermo-epidermal junction, the latter generally producing inconsistent separation *(162)*. The possible saponification of lipids in the hydrated stratum corneum by dissolved ammonia, or the degradation of the tissue components by acid, require consideration with these techniques.

These sampling methods may produce specimens with subtle differences. Histological studies *(158)* have shown the rete ridges of the epidermis are retained intact if the stratum corneum-epidermis is separated from the dermis by heating but are not seen in specimens separated by ethylenediaminetetracetic acid. These preparative methods, therefore, produce membranes having a small, yet possibly significant, difference in overall thickness dependent on the initial presence or absence of dermal invaginations. Furthermore, immersion in hot water rapidly induces hydration of the tissue which may subsequently influence flux data *(29)*. Other methods that have been reported for epidermal-dermal separation include enzymatic *(163)*, or the exposure to dithiothreitol *(164)* or sodium bromide solution *(160,165)*.

Stratum Corneum

In many cases only the stratum corneum is required for experimental work. Proteolytic enzymes may be employed to degrade the viable cells of the epidermis which are then easily removed from the dead horny layer *(28,157,166,167)*. The stratum corneum plus epidermis, previously separated from the dermis, is placed epidermal side down on filter paper soaked in 0.0001% trypsin and 0.5% sodium bicarbonate or buffer solution (pH between 8.0 and 8.6) and incubated at 37 °C for 24 hours after which the digested epidermis may be removed. Other proteolytic enzymes such as pepsin, papain, ficin, elastase or pronase *(168)* may be

used but these are less efficient and require higher concentrations for equivalent effect. Bacterial metabolites such as purified fractions of staphylococcal exfoliatin may also be used to degrade the epidermal cells *(169)*. It must be borne in mind that these enzymes may also affect the proteinaceous content of the stratum corneum and thereby possibly alter its permeability properties. Stretching of the skin sample causes splitting of the strata and may also be used as a method of stratum corneum preparation *(18)*.

There are a number of preparative techniques that may be utilized if excised skin is not available. Sheets of stratum corneum may be removed from volunteers by applying and stripping the skin using adhesive tape *(48,106,145)* which removes approximately 15 μm of stratum corneum with the generation of little discomfort for the donor. Alternatively, intraepidermal blisters may be induced on the skin of volunteers by the controlled application of 0.2% cantharidin solution *(157,162,170)*. Blisters of fairly large diameter (several centimetres) may be produced in this fashion and excised for use in experiments. Cantharidin blisters originate within the epidermal layer and therefore generate very little, if any, scarring of the donor skin on healing of the wound. Alternatively, blisters may be induced by the controlled application of ammonium hydroxide solution, however it has been observed that prolonged exposure of the skin causes destruction of the epidermis and superficial dermis *(171)*.

Suction blisters may be induced by vacuum application to the skin using a manifold device *(172,173)* but stratal separation is more deep-seated and generally disturbs the dermal layers with resultant scar formation. This sampling method appears to be the most appropriate because the degradative effects of chemicals or heating are avoided, and microscopic examination of the excised tissue demonstrates no discernible alteration in the histology of the upper skin layers. However, scar formation makes this an unattractive method for routine sampling. Kiistala and Mustakallio *(172)* report that blisters may be induced on corpse skin by prolonged vacuum application which may be a useful alternative to dermatome excision.

Several workers *(23,157,174)* have reported that there is no significant experimental difference in the *in vitro* performance of stratum corneum samples directly attributable to the method of preparation (cantharidin blister, heat, trypsin, heat plus trypsin or ammonia fume exposure). This does not suggest, however, that the prepared membrane retains the same barrier potential as the unseparated tissue source *(17)*. Conversely, there are numerous reports of the deleterious effects of membrane separation techniques. Several researchers *(157,175,176)* have warned that solutions of trypsin more concentrated than

0.0001% may solubilize part of the fibrous protein matrix of the horny layer. Rietschel and Akers *(162)* report that the most satisfactory method of obtaining stratum corneum appears to be via the cantharidin blister technique. Alternatively, Anderson *et al. (177)* suggest enzymatic preparation on the basis that heating or stretching are more likely to affect the horny layer integrity.

Formulating definite conclusions from these reports is difficult because in many cases the variability between samples harvested by different methods may be masked by the large, inherent interspecimen variability. It must be borne in mind that these are all fairly rigorous procedures that must affect the isolated tissue in some fashion. Good experimental protocol, therefore, would select a process which subjects the stratum corneum to the fewest possible chemicals in lowest effective concentration and avoids extremes of exposure to heat or solvent immersion.

ANIMAL MODELS

Given the limited availability of human tissue and the fact that a number of percutaneous investigations may be too toxic to be carried out on living subjects, a number of animal models have been investigated for their usefulness in predicting percutaneous absorption kinetics. The skin of experimental animals differs markedly from that of humans in features such as thickness and biochemical composition of the stratum corneum, and especially in the density of hair follicles and glands *(178)*. Furthermore, Elias *et al. (154,169)* have suggested that the lipid content of the skin is a major determinant in its barrier potential and that differences between species or between sites are due to varying lipid composition. Even in situations where animal membranes may resemble human tissue (*eg.* "hairless" animal species) direct extrapolation of permeation results to the human situation is not implied.

Skin permeability has been reported by several researchers to increase in the following order: chimpanzee, man, weanling pig, monkey, dog, cat, horse, rabbit, goat, guinea pig and mouse *(48,179,180)*. Generally, *in vitro* data agrees closely with *in vivo* observations in that common laboratory animal skin is more permeable than human tissue, while pig and monkey skin approximate its diffusive barrier and give permeability results that are most comparable *(181)*.

Sampling and stratal separation methods used for human skin may equally be applied to animal membranes with reservations and modifications where necessary. In all cases where sampling of biological tissue is undertaken (both human and

animal) some method of verifying the integrity of the tissue after preparation is essential *(181)*. This procedure is most accurately accomplished by conducting a preliminary permeability investigation using a model diffusing compound, such as tritiated water, and comparing the results obtained to "normal" permeability values for that membrane type *(182)*. Table I lists the summarized permeability coefficients of water and paraquat measured through animal membranes *in vitro (27,78,112,152,183,184)*.

TABLE I: *In vitro* permeability coefficients of paraquat and water for human and animal skin.

Species	Permeability coefficient (cm h^{-1} x 10^5)	
	Water	Paraquat
Human (abdominal)	93-250	0.7
Human (scalp)	950*	-
Human (dermis)	21996*	-
Rat (Wistar)	103	26.7*
Fuzzy rat	110	-
Hairless rat	130	35.5*
Nude rat	152	35.3*
Mouse	144	37.2*
Pig	180	-
Rabbit	253	79.9*
Hairless mouse	351	1066.4*
Guinea pig	442*	195.7*
Dog buccal mucosa	18396*	-

* Significantly different to full thickness human abdominal skin.

These values serve as a useful reference for tritiated water validation and also serve to substantiate the rank order of permeability proposed by Marzulli *(48)* and other workers. Table II lists the thicknesses of the different layers of human and laboratory animal skin as reported by Bronaugh *et al. (178,182)*. Although Kligman *(185)* has suggested that differences between species are due to variations in skin thickness alone, an examination of skin thickness along with other data, such as lipid composition and hair density, may provide more meaningful interpretation of the variance between species *(181,186,187)*.

TABLE II: Human and animal skin thickness measurements.

Species	Stratum corneum(μm)	Epidermis (μm)	Whole skin(mm)
Human	16.8	46.9	2.97
Pig	26.4	65.8	3.43
Rat (female)			
back	18.4	32.1	2.09
abdomen	13.7	34.8	0.93
Rat (male)			
back	34.7	61.1	2.80
abdomen	13.8	30.4	1.66
Hairless mouse	8.9	28.6	0.70
Mouse	5.8	12.6	0.84

Mouse

Laboratory rodents are a convenient source of animal skin for research purposes because these animals are fairly easily handled and are relatively inexpensive when compared with larger species. However, it has generally been

reported *(48,182,184)* that murine skin is more permeable than human tissue, probably due to the thinner stratum corneum, and the medium is, therefore, of limited value.

Hairless Mouse

These laboratory animals have been used extensively in transdermal absorption research, probably because of the perceived similarity between their skin structure and that of humans *(42,78,88,109,110,188)*. Homozygous animals carrying the *hr* hairless, recessive, genes develop a normal coat of hair up to the age of about 10 days after which the complete hair shaft is lost from the follicle. Sparse, thin hairs grow at intervals during the life of the animal but are soon lost. Hyperkeratosis of the stratum corneum and upper part of the hair canals begins about two weeks *post partum* and cysts may develop in the hair follicles or sebaceous glands with subsequent keratinization *(189)*. While the number and diameter of the hair follicles in hairless mouse skin approaches that of human skin more closely than most other laboratory animals *(182)*, the stratum corneum of these animals is less than half as thick as that of human tissue with, presumably, commensurately lower barrier properties. The integrity of the horny layer must be assured if hairless mouse skin is to be used in diffusion experiments *(12,190,191)*. In this regard, the curved toenails of these animals grow excessively long and may inflict substantial injury when males fight.

Reports from the literature tend to suggest that the observed permeability of hairless mouse skin is highly dependent on the characteristics of the permeant, being equivalent to human tissue for some compounds *(107,143,191,192)* and vastly different for others *(182,184,190)*. Stoughton *(192,193)* has investigated the penetration of a number of steroids through human and hairless mouse skin and has found the flux to be in the same range for both skin types, although always higher for the mouse skin (*eg.* betamethasone 17-valerate permeation 1.2-fold greater through hairless mouse skin). Other researchers have found the variance between hairless mouse and human skin to be of greater magnitude but note that the animals are useful for the preliminary screening of drugs for structure-intrinsic diffusivity relationships *(133,134)*.

Behl *et al.* *(190)* have conducted an extensive investigation into the factors affecting diffusion of tritium labelled hydrocortisone through hairless mouse skin *in vitro*. They report that the permeability of the skin varies in a cyclical pattern coincident with the development and loss of fur over the first 35 *post partum* days and, furthermore, that samples of 5- and 35-day old mouse skin exhibit a definite biphasic permeability pattern during

the course of each experiment. These results are significant in that mice of uniform age, preferably older than 35 days, should be selected for diffusion experiments to alleviate any age related permeability variance. These authors report a difference of two logarithmic orders in the permeabilities of human and hairless mouse skin to the permeation of hydrocortisone; a similar variance in permeability may be expected for other corticosteroids.

Behl and coworkers *(190)* further report that the permeabilities of full thickness skin from dorsal and abdominal sites were insignificantly different while stripped skin from the abdomen was found to be more permeable than that from the back. These findings are similar to those from earlier work *(29)* and imply that skin from both anatomical regions may be used interchangeably in diffusion experiments, thereby minimizing wastage of animal skin after sacrifice.

The skin of the hairless mouse is experimentally useful from a sampling viewpoint in that it is loose and does not adhere to the underlying viscera. The facile removal of large pieces of membrane of uniform thickness with little clinging peritoneal tissue is therefore possible. In most cases full thickness hairless mouse skin has been used as separation of the relatively thin stratum corneum-epidermis from underlying tissues by chemical or dermatome means is fairly difficult *(153,158,190,191)*. Histologically, hairless mouse skin does not exhibit the rete ridges of human tissue and hence the thickness of the stratum corneum-epidermis is fairly uniform in whole skin samples.

Valia and Chien *(81,116)* have studied the rates of uptake, binding, cutaneous metabolism and permeation of estradiol in the abdominal epidermis and dermis of hairless mouse skin. No sex-dependent difference was observed in the mechanism and rate of metabolism to estrone, however the uptake and binding of estradiol by female skin was greater than that by male skin. Furthermore, their results imply that there may be a sex-linked difference in the partition coefficient of this diffusant between donor solution and skin. While the bioconversion and binding of corticosteroids may not be as prolific as that of the sex steroids in these animals, the potential for preferential metabolism or preferential partitioning of the diffusant into the skin with regard to the sex of the animal requires consideration. It is generally assumed that animals of the same sex should be used for comparative investigation to overcome the variance introduced by sex-related idiosyncrasies.

The question of simultaneous transport and enzymatic metabolism of permeants in hairless mouse skin has been addressed by several workers *(194, 195)*. Data presented indicates that esterase enzymes leach from the dermal

side of the skin into the receptor phase of diffusion cells, reducing the activity within the membrane by 50% after two hours of contact. These observations are important because the leached enzymes continue to catabolize permeants in the bulk receptor phase and assay of the receptor solution would, therefore, yield a lower value than expected. It has, thus, been proposed that only preleached skin should be used for diffusion experiments, especially those involving permeants which are susceptible to esterase molecular rearrangements. It is believed that a convenient manner of accomplishing this leaching step would be to combine it with the membrane integrity validation procedure using tritiated water as this procedure bathes the dermis for periods longer that two hours.

A further point of interest is that several researchers *(29,77,78,151, 194)* use the same skin sample for repeated diffusion experiments, with intervening saline wash cycles, and report negligible increase in permeability between experiments. Although this may be possible when total diffusion time for any one run does not exceed 60 minutes, the deterioration of the membranes expected over the course of diffusion runs lasting several days, necessary for slowly diffusing species such as corticosteroids, would probably preclude the re-use of the same skin samples.

Rat

The rat is a common laboratory animal which has been used extensively in the study of transdermal drug absorption. Excised rat skin has generally been considered more permeable than human or pig skin *(196,197)*, however in the diffusion of certain compounds rat skin appears to be as good as pig skin in modelling absorption through human tissue *(15,155,166,182)*. Studies using the hairless rat *(92,198)*, the nude rat *(184)* and the fuzzy rat *(153)* have also demonstrated these animals to be useful in certain circumstances.

While split-thickness skin (350 μm), prepared by dermatome, has been used in diffusion experiments *(15,151,153)*, clipped, full thickness skin is more commonly employed *(142)*. The dermatomed sections contain only a portion of the epidermis and demonstrate enhanced absorption of hydrophobic compounds in comparison to full thickness tissue *(82)*. Although adequate correlation of *in vitro* with *in vivo* data has been shown possible, extensive sex and anatomical region-related variations in permeability have been noted for rat skin by other researchers *(178)*. In spite of these observations, the convenience and availability of these animals, and their reasonable similarity in performance to human skin, indicate that this species could play an important role in transdermal absorption research.

Guinea Pig

In contrast to the rat, relatively little use has been made of the guinea pig as a model for transdermal research. Histological studies *(199)* have shown that guinea pig skin is fairly similar in appearance to human skin and one would therefore assume that their *in vitro* permeabilities would be similar. Generally, the clipped, full thickness skin of these animals has been used *(37,149)* and this membrane has demonstrated greater permeability to steroidal *(83,144,200)* and tritiated water *(184)* passage that human tissue.

In most cases, the studies conducted are individual investigations and no correlation to human skin is inferred. However, this does not detract from the usefulness of the guinea pig model as a screening tool for permeation and penetration enhancing effects.

Rabbit

Although good agreement has been shown by Creasey *et al. (201)* between *in vivo* and *in vitro* permeation results of water, rabbit skin is generally recognized as the most permeable of the commonly used laboratory animals *(83,123,183,184,202)*. These findings would suggest that the rabbit is a poor predictive model for transdermal absorption studies. However, rabbit skin is a good indicator of dermal toxicity, because of its rapid absorptive characteristics, and has been used in this mode *(182)*.

Monkey

One may assume that the skin characteristics of primate animals should be very similar to that of humans, making them valuable models in absorption research. Close correlations of human and primate permeation data has been demonstrated by several studies *(48,181,203-206)*. However, these animals require additional holding and laboratory facilities as well as extra care in experimentation which makes their use undesirable *(182)*.

Pig

In several *in vitro* studies the skin of the pig or miniature pig has proven to be a good animal model for human skin *(48,182,197)*. Galey and coworkers *(112)* have observed the permeation rate of tritiated water through dermatomed, full thickness pig skin to be negligibly greater than that through human tissue. Hawkins and Reifenrath *(148)* have reported a statistically

significant correlation between the percutaneous penetration of 10 compounds through whole pig skin *in vitro* and values reported for human skin *in vivo*. They further report that better agreement between *in vitro* and *in vivo* results are obtained if sectioned pig skin is used instead of full thickness membrane, presumably because the barrier thickness of the pig skin would then approach that of human tissue.

Although the stratum corneum of the pig is almost twice the thickness of the human layer, Bronaugh *et al. (182)* report that pig skin is very similar to human tissue in the density of hair follicles (both models average 11 follicles cm^{-2}) and that this is the lowest density found in all the laboratory animals. However, pig skin follicles are almost twice the diameter of their human counterparts and are exemplified by the coarse hair shafts. The thickness of the porcine dermis makes the use of full thickness skin impractical, however Bronaugh *et al. (182)* indicate that they were unable to separate the epidermis from full skin samples by the methods of heat, trypsin or ammonia because of the presence of the coarse hair follicles. They, therefore, conclude that the best method of pig skin preparation is to section the sample using a dermatome, in congruity with the report of Hawkins and Reifenrath *(148)*.

Examining these reports, and bearing in mind the similarities between porcine and human skin, one may assume that the pig is a fairly reliable animal model for predicting percutaneous absorption in man.

Summary

It is evident from the reported literature that no single animal model can mimic percutaneous absorption in man for the diverse range of chemicals which are investigated for clinical and toxicological effects. On the basis of the foregoing reports it is concluded that a useful, initial, screening of animal models should include a member from the rodent family and a larger species (such as the pig). This study has chosen the hairless mouse and weanling pig for preliminary investigation into the *in vitro* permeation of betamethasone 17-valerate. The data from these two animal models, when compared to human skin and synthetic media results, should prove invaluable in assessing the viability of a laboratory diffusion model.

Egg-Shell Membrane

An interesting investigation *(93)* uses egg-shell membrane which, like the human stratum corneum, consists mainly of keratin. The membrane is prepared by immersing the whole egg in 0.5N hydrochloric acid which dissolves the outer

shell. Thereafter, the contents of the egg may be removed and the membrane washed and refrigerated or soaked in isopropyl myristate under vacuum to impregnate the keratin matrix. The replacement of water in the membrane with this lipid is assumed to increase its likeness to stratum corneum biochemistry. It was found that egg-shell membrane was more permeable than cellulose media and exhibited approximately equivalent permeability to a polyamide lipoid membrane for the passage of betamethasone 17-valerate.

Although its relatively low resistivity would tend to suggest that egg-shell membrane has limited usefulness in the design of *in vitro* diffusion systems, the medium has been evaluated in this research for comparison with animal skin data.

SYNTHETIC MEMBRANES

It may, theoretically, be possible to adequately simulate the *in vivo* permeation of a drug using a specific diffusion system and synthetic membrane. The commercial availability, stability, interbatch uniformity and ease of usage makes the use of synthetic media highly desirable and has led to several studies in this field *(75)*. The barrier potential of porous membranes is dictated by the probability of a diffusant molecule entering and diffusing through the pores, and the only factor governing selectivity to diffusion would be the relative molecular size and shape. Conversely, aporous media appear to offer some rate-limiting factor to permeation and, therefore, may more closely simulate the diffusion through biological tissue. The barrier properties here generally relate to the solubility of the diffusant in the polymer matrix and partition coefficient between donor vehicle and membrane.

Cellulose Media

Cellulose is a relatively rigid structure consisting of glucopyranose rings combined by β-1,4-linkages. This conformation allows only two types of movement in the chains: inversion of the pyranose ring ("chair" to "boat" forms) or rotation around the glycosidic linkage. In addition, the cellulose chains exist in a partially crystallized form due to interchain hydrogen bonding *(75)*. Commercial cellulose membranes have a cut off of 8000-15000 daltons for molecular dialysis and on purchase normally contain a number of softener, preservative and plasticizer additives which may affect drug permeation depending on the membrane pretreatment prior to experimentation. Cellulose acetate (dialysis) media has been used extensively in diffusion cell systems

(24,31,32,65,69,207-211), while cellulose nitrate has been used as a model for the gastric barrier *(212)*.

Corrigan *et al. (210)* have used cellulose dialysis tubing as a diffusion medium for hydrocortisone and its polyvinylpyrrolidone coprecipitates. Pretreatment of the membrane here involved distilled water washing to remove water and sulphur compounds and soaking in water to allow swelling.

Barry and coworkers *(13,97)* have investigated the *in vitro* diffusion of a number of radiolabelled corticosteroids through cellulose acetate media using a bichamber steady-state diffusion apparatus. Washing in warm water and storage in cold water for one week more than doubled the thickness of the membrane and it is through this aqueous environment that diffusant molecules are thought to diffuse. However, measured diffusivities were 25-fold smaller than calculated aqueous mobility and it is suggested that this is mainly due to diffusant bonding interaction with the cellulose polymer. From this report it can be summarized that the diffusion coefficient of steroids in cellulose media is: independent of donor concentration (provided this is relatively dilute), dependent on temperature, independent of polarity at low temperatures for molecules of similar size and shape, and increases disproportionately at higher temperatures for polar steroids to values greater than expected by normal kinetic theory. Generally, cellulose membranes are reported to be more permeable than biological membranes or aporous synthetic media *(108)* and are nondiscriminatory to the characteristics of the diffusant molecule.

Filter Membranes

Porous filter membranes have seen relatively little usage in diffusion systems in comparison to the synthetic polymers *(44,213-215)*. Turakka *et al. (64,71)* have used a polycarbonate filter membrane to separate topical delivery formulations from receptor media in a simple drug release apparatus. Interestingly, they state that this membrane was chosen for investigation in preference to cellulose or silicone media because hydrocortisone acetate was not found to diffuse through the latter two membranes into propylene glycol receptor phase using their particular diffusion cell.

Generally, porous filter media appear to be most useful as a dividing medium or as supporting screens where the release rate of drug from the delivery system is under investigation, and not the actual transdermal kinetics of the permeant. In these cases the filter medium does not simulate the skin and provides no significant barrier to diffusant passage.

Synthetic Polymers

Diffusion of a molecule through continuous synthetic polymer is analogous in many ways to diffusion through unstirred liquids. Mass transfer through the matrix is dependent on the frequency of void formation of sufficient size to accommodate the diffusant. Voids are formed by the random oscillation of polymer chains and the larger the diffusant species the greater the number of neighbouring polymer units which would have to move in a specific manner in order to generate a void of sufficient volume to accommodate the diffusant. The degree of bonding interaction between the polymer chains will determine the rigidity of the matrix and, thus, the propensity for hole formation and resultant permeability *(123,126)*. Furthermore, crystallinity within the matrix generates regions of low diffusivity and the presence of solvents facilitates the oscillation of polymeric segments, both situations altering the overall permeability of the membrane.

A number of studies have been carried out using synthetic polymers as diffusion media *(52,100,188)*. The diffusion characteristics of various steroids through hydrogel films composed of hydroxyethyl methacrylate have been reported by Zentner *et al. (216)* and other workers *(75)*.

Silicone polymers such as polydimethylsiloxane have received recent acclaim because they are lipophilic in nature and highly permeable to many non-ionic drugs which dissolve in the barrier matrix and diffuse across it *(40,74, 90,101,108,111,125,207,217-222)*. Steroidal diffusion in silicone polymers may occur mainly via solution interaction with the polymer chains and not through bulk fluid pores *(218)*. Lee *et al. (123,124)* have studied the effect of varying polymer backbone structure on the permeation of progesterone and testosterone. They synthesized a number of polydimethylsiloxane membranes in which each alternate bonding oxygen atom between polymeric units was replaced with an organic constituent. Generally, the permeability coefficients of the diffusants varied inversely with the rigidity of the synthesized polymers. Roseman *(223)* has reported diffusion coefficients of the same order of magnitude for a number of different progesterone-like steroids studied. This is expected for molecules of essentially similar size and conformation if one assumes the coefficient is dependent on the ease of molecular movement between the mobile polymer chains. An apparently more important variable affecting the permeation process is the steroid partition coefficient between donor or receptor vehicle and membrane, as is the polymer solubility of the drug *(16, 126,223)*. These two factors are highly dependent on structure and even small molecular modifications may greatly alter partitioning and solubility *(126)*.

Alternatively, Di Colo *et al. (224)* have observed that prednisolone is released faster from silicone matrices containing glycerol or polyethylene glycol, which may suggest that the interchain water environment is involved in the diffusion mechanism. Carelli *et al. (225)* conclude that drug movement is controlled by a composite dissolution-diffusion mechanism. Diffusant molecules in the environment of the interpolymer fluid are most mobile and are cleared first from the medium while a certain fraction of the diffusant species are dissolved in or surrounded by the polymer and are, therefore, adsorbed to a greater extent.

Commercial products may contain additive fillers (*eg.* 20-30% silica or graphite) to improve the membrane strength and their presence may increase the barrier potential to permeation. This filler is randomly orientated, non-uniform in size and is impervious to permeants but readily enters into adsorption interactions. Permeation rates are, therefore, generally lower through filled media *(95,102,126,225)* and lag times are increased *(218,223)*. Generally, it is concluded that these fillers simply reduce the volume of polymer which is available for the steady-state passage of permeating molecules and makes their diffusive path longer and more tortuous. Silicone membranes without additives are currently available and their choice for diffusion cell research is obviously advantageous.

Therefore, the most important parameters with regard to permeation appear to be polymer chain mobility (rigidity) and the solubility of the diffusant in the matrix (which influences partition coefficient). Generally, it is assumed that silicone membrane is the most useful of the synthetic media for use in diffusion cell systems. Its relatively inert, lipophilic nature makes it an ideal environment for partitioning and permeation of lipophilic drugs while its aporosity provides some rate-limiting function to this process.

2.3 DIFFUSION CELL DESIGN VALIDATION

The foregoing accounts of diffusion cell design and membrane selection exemplify the formidable task in the choice of a model for *in vitro* diffusion studies. The multitude of cell variables such as temperature, degree of agitation, sampling procedure, chamber concentrations and permeant solubility, must be provided for, or controlled precisely, before the problems of membrane area, thickness, integrity and biological properties are considered. Overall, a design equilibrium must be achieved between the variables and controls so that accuracy and precision are maximized. Care exercised in the design and

evaluation of a new *in vitro* diffusion cell system will obviously enhance the credibility of the results obtained. Any new system, therefore, initially requires a complete analysis of its performance with respect to the common *in vitro* variables. Only once this has been accomplished, and satisfactory results obtained, may experimental permeation data be reported with any degree of confidence.

The major problem envisaged with most of the cell designs reported in the literature is the absence, inadequacy or non-uniformity of fluid agitation within the chambers. Other problems that may be experienced, but are seldom mentioned, include air bubble formation on the underside of the membrane (that may be difficult to dislodge once the diffusion run has been initiated), the presence of a hydrostatic fluid pressure may cause the membrane to distend, or obtaining a uniform seal around the membrane flange may also pose a problem. The following, summarized, features may be proposed as those of an ideal *in vitro* diffusion cell system:

1. An optimal diffusion area to receptor volume ratio which will allow sensitive permeant analysis in the receptor medium, especially at early sampling times when permeant concentrations are low. The proviso here is that the diffusion orifice, and the surrounding membrane-securing flange, should accommodate the smallest area of biological tissue usually obtained by the particular sampling technique employed. Large sheets of synthetic media are in ample supply and they, obviously, will not dictate the diffusion area incorporated into the cell.
2. Homogeneous fluid mixing must be generated throughout the chambers (at the membrane surface, in the bulk fluid and within the sampling arm) and should be sufficiently vigorous to minimize diffusion boundary layers. This would require an internal, mechanical mixing procedure in contrast to simple shaking of the cell.
3. The cell design must support rapid temperature equilibration within the chambers so that gradients are not established between the bulk cell contents and the membrane interface. Adequate thermal control would probably require total thermostatic envelopment of the cell chambers and membrane region, either by complete immersion of the cell in a water bath or total water jacketing of the chambers and flange.
4. The conformation of the diffusion cell chambers should be as uniform as possible, with a minimum of appendages and no physical constrictions between the agitated bulk cell fluid and the membrane surface.
5. The sampling port should form part of the cell body and should be attached in such a fashion as to facilitate adequate mixing of its contents with

the bulk chamber fluid. The port should not contain a relatively large proportion of the chamber fluid and should have some stopper system to prevent evaporation of the chamber contents. A hydrostatic fluid pressure should not be generated on the membrane, and the port should, ideally, also allow the facile expulsion of any air bubbles that may form and become trapped on the undersurface of the membrane.

6. The cell design should be easily constructed using basic laboratory materials. Glass is the ideal construction material as it is inexpensive, easily worked into any idealized conformation, is almost totally inert to the normal laboratory chemicals, and supports rapid thermal conduction. Careful design and planning may incorporate other basic laboratory equipment into the diffusion cell system, such as thermostated water baths or magnetic stirrers, thereby further simplifying construction and reducing cost without sacrificing sensitivity in the monitoring of permeation.
7. Optimal design would also produce a cell that is versatile in performance: one that may be used for both steady-state and *in vivo*-mimic, finite and infinite dose diffusion experiments.
8. A rate-limiting, discriminating membrane appears to be essential for demonstrating subtle differences between the drug release characteristics of similar topical formulations. Equally, a relatively innocuous solvent, such as isopropyl myristate, is favoured as the receptor phase because of its relatively ideal solubility and partitioning characteristics as well as its biochemical similarity to the physiological environment of the skin.

Although these features may represent the ideal diffusion cell design, the incorporation of all these facets into a single system may be impractical, however as many of them as possible should be included into any new cell proposed for study. In addition, it has clearly been established *(15,151-153)* that flow-through cell designs exhibit no advantage over static designs in respect of the permeation kinetics obtained, if the conformation, receptor medium and agitation of the static systems have been optimized. Bearing in mind the additional cost of construction and ancillary equipment required for the flow-through cells, the choice of a static system is appropriate if sufficient attention is applied to the foregoing details.

NEW *IN VITRO* CELL DESIGN

Figures 8 and 9, and Plates 1 and 2, indicate the design of the new *in vitro* cell used in the steady-state and *in vivo*-mimic experimental modes, respectively. In either the horizontal or vertical mounting configurations,

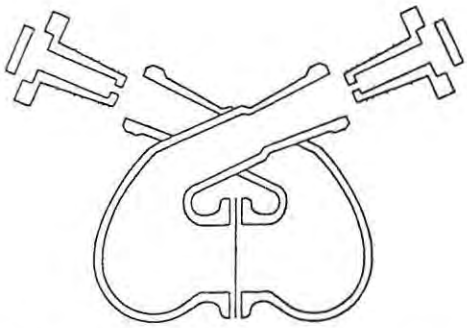


FIGURE 8: *In vitro* diffusion cell orientation for steady-state permeation experiments.

PLATE 1: *In vitro* cells in laboratory clamp for steady-state permeation experiments.

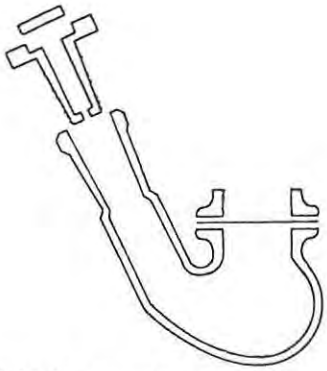


FIGURE 9: *In vitro* diffusion cell orientation for *in vivo*-mimic permeation.

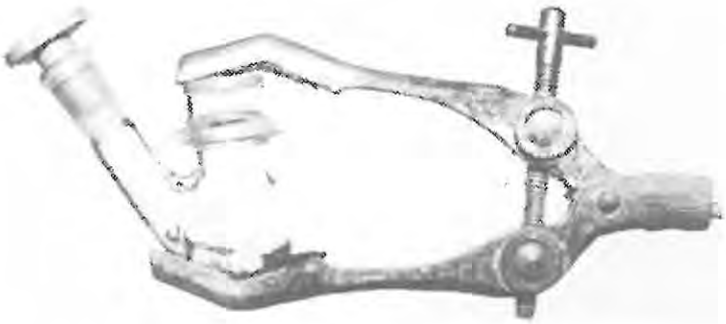


PLATE 2: *In vitro* cells in laboratory clamp for *in vivo*-mimic permeation experiments.

fluid is filled into the chamber to the level of the membrane so that no distending stress is applied to the medium. The glass chambers are constructed as spherical as possible which, it is assumed, will generate the most efficient hydrodynamic fluid mixing. The diffusion orifice forms part of the wall of the chamber so that there are no cylindrical constrictions between the bulk cell body and the membrane surface to impair fluid movement. Appendages fixed to these spheres are minimized: a single, inclined sampling port allows introduction of the chamber fluid and extraction of samples during the experiment. The diameter of this sampling port is relatively large, approaching the diameter of the spherical chamber, so that fluid mixing and mass transfer into the port are easily achieved, while ensuring that a relatively small percentage of the total fluid volume is contained within these ports. The mouths of the sampling ports have regular 10 mm internal ground glass faces which will accommodate modified teflon stoppers. These stoppers are hollow and each has a 2 mm diameter hole drilled through its transverse sealing face to allow insertion of the glass tube from a transfer pipette for chamber fluid sampling. In this manner the internal chamber equilibrium is not disrupted during

sampling by total removal of the stopper. This is especially important if volatile fluids are used as substantial evaporative loss of these heated agents may be experienced by repeated stopper removal over several days of experimentation. The stoppers have an outer recess to accommodate a sealing teflon disc between sample extractions. The hole drilled into the stopper is of further benefit in that it allows ambient air to escape from the chamber on insertion of the stopper into the sampling port mouth. Otherwise, this air would be trapped by the stopper and pressurized, increasing the stress on the chamber fluid, distending, and probably rupturing, the delicate membrane.

The internal diameter of the ground glass flange, which secures the membrane in position, is approximately 1.45 cm producing a membrane diffusion area of 1.65 cm^2. This diameter is appropriate for the area of human stratum corneum samples obtained as described in Section 3.3. The other biological tissues employed in this study were in more abundant supply and, therefore, the dimensions of the diffusion area were dictated by the size of the available human tissue samples. This area, coupled with the receptor chamber volume of approximately 10 ml, allowed adequate concentrations of betamethasone 17-valerate to accumulate for analysis by high-performance liquid chromatography.

Fluid mixing is efficiently generated by internal teflon-coated bar magnets using laboratory magnetic stirrers and this agitation maintains homogeneous mass transfer of permeant from the membrane surface to the bulk fluid and minimizes diffusion boundary layers. The revolution speed of the stirrers is calibrated using a custom designed, electronic, magnetic-field induction measuring instrument. These features were easily incorporated into the cell design and were considered more advantageous than the use of externally driven impellers, which would require motorized drive shafts to penetrate the chamber walls. Not only would these shafts create a fluid sealing problem, but they would also preclude both the horizontal and vertical mounting of the diffusion cells. The use of internal bar stirrers allows versatile mounting of the cells in either configuration for steady-state or *in vivo*-mimic experimentation.

Thermal control, in either mounting configuration, is easily achieved by total immersion of the cell in a thermostated water bath. The cell may be lowered into the water to the level of the membrane so that no temperature gradient exists between the bulk fluid and the membrane interface. The glass construction allows rapid equilibration and the avoidance of internal physical constrictions facilitates rapid and homogeneous thermal transfer. In this fashion it is believed that control is more easily accomplished than if the cells were individually water jacketed, not only because of the pump and tubing required for the latter, but also due to difficulties in extending the jacket to the membrane-flange region.

Simple laboratory clamps are used to suspend the diffusion cells in the water bath, which rests on the magnetic stirrers (see Plate 3). Large teflon bar magnets are placed into the water bath, above the stirrers, to assist in the heated water circulation and also to augment the magnetic force driving the smaller bar magnets within the cells.

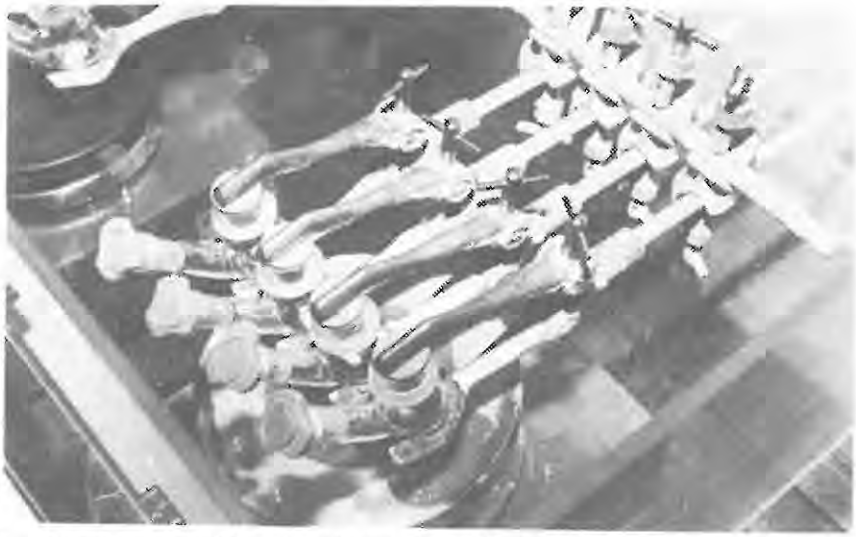


PLATE 3: Arrangement of four *in vivo*-mimic cells in water bath above magnetic stirrer.

By incorporating these features into the new diffusion cell it was hoped that a sensitive system would be established that would accurately and precisely monitor the permeation of betamethasone 17-valerate through various membranes. One further objective in the planning was that the entire system should be established by the judicious incorporation of as many relatively simple common laboratory components as possible. It is believed that the use of complex, sophisticated apparatus, or the application of theoretical correction models, is not a prerequisite for the acquisition of valid data and this has been exemplified by the results from this relatively simple diffusion cell design.

VALIDATION PROCEDURE

Initially the validation procedure for the cell performance and sensitivity involved a number of repeated, steady-state diffusion experiments in which only the degree of receptor phase agitation varied. As the agitation increases so the diffusion boundary layers juxtaposed to the distal surface of the membrane are disrupted and dissipated to a greater extent. The magnitude of this dissipation will increase with stirring speed to a certain, limiting value, above which further increases would insignificantly affect boundary layer thickness. The observed permeation rate would, therefore, initially increase with stirring speed, followed by a constant, plateau flux value at optimal agitation.

Silicone membrane was chosen as a model medium for the stirring speed experiments because of its nonporous uniformity. Betamethasone 17-valerate solution (0.1% in isopropyl myristate) was used as the donor vehicle and permeation to purified isopropyl myristate solvent was monitored at receptor phase stirring speeds at intervals between 0 and 1150 rpm, the cells being maintained at 35 °C.

The effect of temperature (as the only variable) on observed permeation rate was also investigated and the results were expected to behave in a typical Arrhenius fashion (greater permeation at higher temperature). Steady-state permeation experiments (0.1% corticosteroid in isopropyl myristate donor solution to isopropyl myristate receptor solvent) were conducted at optimal agitation speed, as determined by the previous study, while varying the temperature of the system at intervals between 20 °C and 40 °C.

In this manner adequate performance of the cell was verified prior to the commencement of the permeation experiments. It was believed that satisfactory hydrodynamic fluid mixing would adequately be demonstrated by the agitation speed validations. Thereby, it would be assured that diffusion boundary layers are minimized at the membrane interface without the need to quantify the magnitude of these layers. Furthermore, the permeation rates measured at different experimental temperatures would adequately indicate the thermal performance of the system and measured thermal equilibration times would indicate the efficiency of heat transfer throughout the cell. By optimizing the operating conditions of the system in this fashion the results obtained from further investigation would be appropriately reliable and accurate.

this reaction is incapable of qualifying or quantifying steroid degradation products which may therefore be mistaken for parent steroid when the total absorbance is read *(233,234)*. These aspects, when considered with the problems associated with polarographic analysis *(235,236)* and derivatization in gas chromatographic methods *(233,237,238)* makes liquid chromatography the assay technique of choice for the determination of steroids in complex matrices *(238-240)*. High-performance liquid chromatography is selective, precise, accurate and has the potential for simultaneously assaying multiple components, including degradation products and preservatives.

A major problem encountered in topical dosage form analysis by various techniques is the diverse chromatographic interference due to formulation adjuvents present in these relatively complex formulations *(241)*. The majority of formulation components do not appreciably absorb UV light at the normal detection wavelengths employed in analysis and therefore do not appear to alter the chromatographic behaviour of the analytical column during the initial injections *(242,243)*. However, their adsorption to the analytical column packing material after repeated injections would certainly influence the resolution of the more mobile species. Furthermore, these lipophilic components tend to generate gross baseline shift on the chromatogram when they are eventually eluted from the column.

The corticosteroid components, in typical concentrations of 0.025-1%, are presented in relatively complex matrices of vastly differing hydrophilic, lipophilic, emulgent and preservative constituents. Generally, only one or two components require quantitative analysis and these must be adequately separated from the other vehicle excipients which may interfere with the assay process. Prior to chromatographic analysis, therefore, an extraction or clean-up step is normally required to remove the majority of these adjuvents from the samples. This has usually been carried out by a liquid-liquid extraction system for topical formulations containing betamethasone 17-valerate *(243-246)* or other corticosteroids *(229,231,238,239,247)*. Other extraction procedures which have been reported include alcoholic extraction *(248)*, thin layer chromatography *(249-251)*, column chromatography *(227)* or silica gel column separation *(252,253)*. However, all these methods are laborious, time consuming and generally require large samples of the dosage form *(245)*. Obviously, minimizing the amount of sample handling and solvent use prior to chromatography will increase the accuracy and precision of the technique as well as decreasing the overall time and cost of analysis.

It therefore seems logical that a high-performance liquid chromatographic system which effects the simultaneous clean-up of the formulation matrix and quantitative analysis of the steroidal moiety would be the most useful. The

3. EXPERIMENTAL AND ANALYTICAL TECHNIQUES

3.1 TOPICAL DOSAGE FORM ASSAY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The quantitative measurement of corticosteroid diffusion through *in vitro* membrane systems necessitates the use of several ancillary analytical techniques. One variable that must be controlled closely is the mass of diffusant applied to the donor surface of the membrane as this may markedly affect the magnitude of the flux observed. If simple solutions are used as the donor vehicle then quantifying the concentration of permeant is a fairly facile process. Moreover, if the proper care and attention is applied in the preparation of solutions in the laboratory then assay of these vehicles prior to experimentation is generally considered unnecessary.

A problem arises when commercial products are used for research purposes. In many instances the concentration of drug in the purchased topical formulations is not exactly the label specified value, due to factors such as overage addition by the manufacturer, degradation or container adsorption. It is possible that two formulations may vary in concentration by 20% and still not exceed the pharmacopoeial acceptable limits for content strength. If these formulations are accepted as label strength then misleading conclusions concerning the drug release characteristics of the vehicle may be drawn from the results of *in vitro* or *in vivo* assays, whereas discrepancies in performance are actually due to drug concentration differences. It is therefore imperative that a reliable, analytical technique be available so that the exact drug content of all vehicles used in diffusion research may be measured. Only in this manner may conclusions be made from flux data with some degree of validity and confidence.

BACKGROUND

Classically, the quantitative analysis of corticosteroids has been carried out by colourimetric assay by reacting either triphenyltetrazolium chloride or tetrazolium blue with the C-17 α-ketol steroidal side chain under basic conditions *(14,226,227)*. The tetrazolium moiety is reduced quantitatively to a red formazan compound, the intensity of which can be measured spectrophotometrically at approximately 550 nm. However, colour formation is not steroid specific and is widely influenced by a number of experimental conditions and by the presence of topical formulation excipients *(228-232)*. Furthermore,

analytical column would certainly carry out this clean-up procedure. Various techniques have been reported *(229,230,233,246,254,255)* where samples of creams or ointments have been totally or partially dissolved in various solvent systems and aliquots injected directly on to the analytical chromatography column without any additional clean-up procedure. Although this is the most facile method of sample handling, it is assumed that the lipophilic material which adsorbs to the column would adversely affect peak shape and resolution. Although Amin and Schneider *(238)* report that they did not observe formulation components accumulating on the analytical column, and Van de Vaart and coworkers *(242)* report that they experienced no change in base line, peak shape or retention behaviour after repeated injections in this fashion, Olson *(232)* and Beyer *(240)* have reported some loss of resolution due to cream excipients passing through and depositing on the column. In most of the reports in the literature no mention is made of column integrity after repeated sample injections although precautionary intermediate column washes with methanol are often prescribed *(242,255)*. It is therefore assumed that some form of sample clean-up is essential to limit the amount of lipophilic material, especially liquid paraffin *(255)*, entering the analytical column.

Multicolumn high-performance liquid chromatography, utilizing column switching procedures, are highly suitable for the separation and analysis of multicomponent samples, especially those formulated in complex matrices *(256-259)*. Using this technique the sample is dissolved or dispersed in a suitable solvent and an aliquot injected directly on to the high-performance liquid chromatography system which consists of at least two columns. The technique requires that specific fractions of the chromatogram be physically directed either to the detector or to waste. A front cutting technique *(260,261)* is usually utilized in which only the first fraction of the eluent from a pre-column is selectively transferred to the analytical column for further resolution of solutes and subsequent detection. The nonpolar excipients retained on the precolumn are then isolated from the analytical system using a specially designed switching valve and these constituents are flushed to waste using a separate solvent delivery system, preferably flushing in the opposite direction to mobile phase flow *(260)*. Lipophilic component extraction from the sample is thereby effected with minimal influence on the analytical system. Multicolumn high-performance liquid chromatography is thus a highly suitable procedure as it is more convenient, precise, quantitatively reproducible and less time consuming than the use of classical clean-up methods *(261)*.

It was on the basis of this technology that a stability-indicating high-

performance liquid chromatographic assay for betamethasone 17-valerate and its principal degradation product, betamethasone 21-valerate, in cream, lotion, ointment and scalp application dosage forms was developed *(262)*. The new method uses a switching, multicolumn on-line sample clean-up approach which is preferable to the previously reported techniques.

MATERIALS AND METHODS

Reagents And Chemicals

The acetonitrile, methanol and tetrahydrofuran used in this assay procedure were all glass distilled and spectral grade (Burdick and Jackson, U.S.A.). The high-performance liquid chromatographic grade water used in the mobile phase was purified through a Milli-Q system (Millipore, U.S.A.). The mobile phase was filtered through a 0.45 µm membrane filter (type BD, Millipore, U.S.A.) and simultaneously degassed under vacuum at room temperature. All chemicals used were analytical reagent grade and were used as received. The betamethasone 17-valerate was obtained from Glaxo, South Africa; the norethisterone from Ethnor, South Africa; and the betamethasone 21-valerate and hydrocortisone were authentic specimens from the British Pharmacopoeia Commission, U.K.

Apparatus

The high-performance liquid chromatograph consisted of a solvent delivery system (Model M45, Waters, U.S.A.), a syringe loading, six-port sample injector (Model 7125, Rheodyne, U.S.A.) equipped with a loop column (MPLC 3 cm x 4.6 mm i.d., RP-18 packed with Lichrosorb, Brownlee, U.S.A.) and a diode-array UV detector (Model 1040A, Hewlett Packard, U.S.A.) coupled to either a strip chart recorder (Model 100A, Perkin Elmer, U.S.A.) or an integrator (Model 3390A, Hewlett Packard, U.S.A.) set to measure peak height ratios. A second solvent delivery system (Model 6000A, Waters, U.S.A.), capable of solvent switching, was connected to the injector for loop column back-flushing. The analytical column (25 cm x 4.6 mm i.d.) was custom packed with octadecyl-silane packing material (Techsil 10 µm, HPLC Technology, U.K.).

The loop column in this case was found to be of high efficiency and generated insignificant broadening of the elution profiles. This is important as low efficiency precolumns limit the ultimate resolution and sensitivity of the multidimensional technique.

HPLC Conditions

The mobile phase was prepared by adding 45 parts by volume of water to 55 parts by volume of acetonitrile in a stoppered flask and allowing the mixture to equilibrate to room temperature prior to filtering and degassing. There was no ionic or buffer addition to this solution. The mobile phase flow rate was set at 1.5 ml min^{-1} and all chromatography was carried out at ambient temperature. Fluctuations in ambient temperature were not manifest by variations in peak resolution or retention time. The detector wavelength used was 239 nm, bandwidth 30 nm, with a reference wavelength of 330 nm, bandwidth 30 nm. This mobile phase combination of acetonitrile/water was found to be optimum for the resolution of betamethasone 17-valerate from its C-21 ester degradation product. Solutions of methanol in water were investigated and were found to be incapable of adequately resolving the two congeners. Similarly, the addition of methanol fractions to the above mobile phase composition worsened the resolution of these two compounds. This situation may be unique to the particular analytical column used but is readily explained by reference to the results of Munson and Wilson *(263)*. They conclude from their research that the separation of steroids on octadecylsilane columns may not be due entirely to partitioning mechanisms and that adsorption phenomena may play a major role. The packing material of these columns may contain numerous free silanol groups which could interact by hydrogen bonding with the mobile phase or analytes. If methanol/water mobile phases are used, it is proposed that these potential binding sites are saturated by the solvent molecules, which have a high capacity for hydrogen bonding. Analyte molecules must therefore compete for these silanol binding sites resulting in greatly reduced adsorption capacities for these moieties which, for steroids, have relatively low capacity for hydrogen bonding. The overall effect of this methanol swamping of the binding sites is a decrease in the ability of the column to discriminately bond similar molecules (such as the betamethasone esters) to differing extents, with a resultant loss in chromatographic resolution. On the other hand, acetonitrile is an aprotic solvent and does not participate in hydrogen bonding. Analyte adsorption on to the silanol hydroxyl groups will, thus, not be decreased because of interaction with this solvent. Total separation of the corticosteroid esters is therefore possible using acetonitrile/water as mobile phase.

Internal Standard Solutions

Under the operating conditions described above, it was found that the high-performance liquid chromatography system adequately resolved hydrocortisone (2.6 min), fluocinolone acetonide (3.3 min), norethisterone (4.4 min), betamethasone 17-valerate (5.9 min), betamethasone 21-valerate (7.3 min) and medroxyprogesterone acetate (8.8 min). Hence, any one of these compounds could, theoretically, be used as the internal standard for the assay of any other.

Possible detection interference of internal standard could originate from elution of formulation ingredients, especially preservatives. These would be predominantly hydrophilic and would easily elute with the mobile phase composition used. The elution behaviour of the preservative chlorocresol, contained in the cream formulations, and methyl hydroxy benzoate, contained in the lotion formulations, was obtained using the chromatographic equipment and conditions described above. From the relative elution times of these solutes the appropriate choice of internal standard could be made for each topical formulation assayed so that the recorded peaks from preservative and internal standard eluents were not superimposed.

The ointment and topical solution dosage forms contained no preservatives, however, the final injection solutions of both these formulations contained large fractions of organic solvent (tetrahydrofuran or ethanol respectively) which eluted early in the chromatogram. These fairly large peaks prevented the use of the early eluting hydrocortisone or fluocinolone acetonide as internal standards. Hydrocortisone was chosen as internal standard for use in the assay of the cream dosage form and norethisterone for the lotion, ointment and topical application assay.

Calibration Standard Solutions

Standard stock solutions of betamethasone 17-valerate were prepared in acetonitrile at concentrations of 2.5, 5.0, 10.0, 12.5 and 20.0 $\mu g\ ml^{-1}$. Each solution contained either 3.0 $\mu g\ ml^{-1}$ hydrocortisone or 3.5 $\mu g\ ml^{-1}$ norethisterone as the internal standards. These internal standard concentrations would yield approximately equivalent peak heights to that expected from the label concentration of betamethasone 17-valerate contained in the formulations. Similarly, in order to quantitate the degradation product, betamethasone 21-valerate solutions were prepared in acetonitrile at concentrations of 0.5, 1.0, 1.5 and 2.1 $\mu g\ ml^{-1}$, each solution containing the relevant internal standard (3.0 $\mu g\ ml^{-1}$ hydrocortisone or 3.5 $\mu g\ ml^{-1}$ norethisterone).

Sample Preparation

Approximately one gramme of cream or ointment was extruded from its proprietary tube and discarded. Thereafter, a sample was loaded into a 1 ml disposable, plastic syringe (Medispo, South Africa) immediately prior to sample weighing. Wide bore needles were fitted to the syringes through which the formulation samples were extruded. Adsorption of betamethasone 17-valerate on to the syringe material was demonstrated to be negligible by a separate investigation. Samples of lotion or topical application formulations were delivered directly from their proprietary containers without the use of syringes. Additional care was required in the sampling of the topical application because of its alcohol base which made the formulation volatile. This necessitated the use of stoppered containers to prevent weighing errors due to evaporation. Multiple, accurately weighed samples of cream, lotion or topical solution (250 mg) were dissolved in 10 ml of acetonitrile in a 25 ml volumetric flask, which served as the weighing vessel. Ointment samples (250 mg) were dissolved in tetrahydrofuran. Solubilization of the formulation was aided by swirling the flask in a stream of warm air. The flasks were then allowed to equilibrate to room temperature and an aliquot of the appropriate internal standard solution was added. Cream, lotion and topical application solutions were made up to volume with acetonitrile and ointment solutions were made up to volume with tetrahydrofuran.

Tetrahydrofuran was found to be fairly disruptive on the equilibrium of the chromatographic system, probably due to its powerful solvating capacity. Large injection volumes of tetrahydrofuran generated a small loss in peak resolution, presumably due to enhanced migration of the drug fractions through the column under the influence of this strong eluent; observations which have been noted by other researchers *(258,259,264)*. These problems were minimized if the initial ointment solutions in tetrahydrofuran were made up to volume with acetonitrile and just enough tetrahydrofuran to maintain the formulation in solution. This would maintain the tetrahydrofuran fraction in the final injection solution as dilute as possible.

Aliquots of these solutions were filtered (Millex SR, 0.45 µm, Millipore, U.S.A.) prior to injection into the chromatography system. Based on the product label concentration of 0.1% betamethasone, sample preparation as detailed above produced a final betamethasone 17-valerate injection solution concentration of approximately 12.1 µg ml^{-1} (equivalent to a column loading of 121.4 ng), together with either 3.0 µg ml^{-1} hydrocortisone or 3.5 µg ml^{-1} norethisterone as internal standard.

Injection Procedure

A 10 µl-volume of sample or calibration standard solution was injected into the valve (Figure 10) placed in the "load" position (off-line). Without removing the syringe the injector valve was turned to the "inject" position (on-line), thus introducing the sample aliquot into the chromatographic system. The syringe was then withdrawn and the valve left in the inject position for three minutes to allow only the components of interest to pass through the loop column and transfer to the analytical column. The injector was then turned back to the load position. In the case of sample injection this procedure trapped lipophilic formulation excipients on the loop column. These formulation adjuvents were back-flushed off the loop column using 1 ml of methanol followed by 5 ml of mobile phase which was pumped using the second solvent delivery system at a flow rate of 2 ml min^{-1}. In this manner no re-equilibration of the loop column with the remainder of the analytical system was required prior to the next injection.

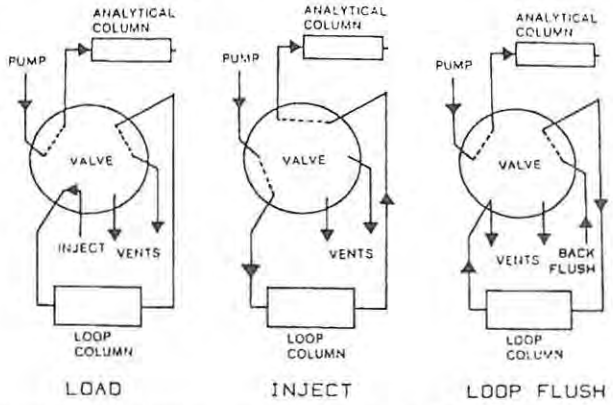


FIGURE 10: Sequential schematic diagram of injection valve positioning during assay showing solvent elution direction at each stage.

Both methanol and tetrahydrofuran have been used as the initial solvent in this back-flushing process *(257,258)*. It was found that methanol aliquots were adequate for displacing bound formulation adjuvents from the loop column, probably due to preferential hydrogen bonding capacity with the packing silanol groups as discussed previously. Furthermore, the disruptive effect that tetrahydrofuran had on the system equilibrium made its choice undesirable.

Calibration standard solution injections were interspaced between the sample injections, ensuring that each solution was injected at least three times for means to be calculated. Injector switching and the back-flushing procedure were omitted when the calibration standard solutions were injected as these solutions contained no lipophilic material. Separate investigation showed that the omission of these manoeuvres had no effect on the peak heights obtained. Peak height ratios of injected samples could be compared with ratios from calibration standard solutions in order to assign a value to the

mass of corticosteroid present in the sample aliquot.

Calculations

All determinations of corticosteroid content from the resultant chromatograms were carried out using the relative response factor (RRF) method *(265, 266)*. This manipulation accounts for the effects that daily operation variables, such as mobile phase preparation or ambient temperature, may have on resolution and peak shape. The RRF was calculated daily using the means of the measured peak heights from the calibration standard solution injections in accordance with Equation 13:

$$RRF = PH_{is} \times MASS_b / PH_b \times MASS_{is} \qquad \text{Equation 13}$$

where PH_{is} and PH_b are the peak heights of internal standard and betamethasone 17-valerate respectively, and $MASS_{is}$ and $MASS_b$ are the respective masses of internal standard and betamethasone 17-valerate in the standard calibration solutions. The mass of corticosteroid in each formulation sample aliquot could then be calculated using Equation 14:

$$MASS_{bs} = RRF \times PH_{bs} \times MASS_{is} / PH_{is} \qquad \text{Equation 14}$$

where PH_{is} and PH_{bs} are the peak heights of internal standard and betamethasone 17-valerate respectively, and $MASS_{is}$ and $MASS_{bs}$ are the respective masses of internal standard and betamethasone 17-valerate in the sample solution.

RESULTS

A typical chromatogram of a betamethasone 17-valerate containing cream sample is shown in Figure 11 and depicts the resolution between the preservative (chlorocresol) and the internal standard (hydrocortisone). Figure 12 is a chromatogram of a typical lotion sample including the norethisterone internal standard and demonstrates the large absorption peak for the methyl hydroxy benzoate preservative. Figure 13 shows a chromatogram of an ointment sample including the norethisterone internal standard and the very large solvent front caused by the tetrahydrofuran. The twin peaks of the solvent front have not been individually identified but are presumed to be oxidative byproducts of the tetrahydrofuran solvent which tends to form etherial compounds on exposure to air. The chromatographic trace of a scalp application sample is very similar to that of the ointment and is shown in Figure 14.

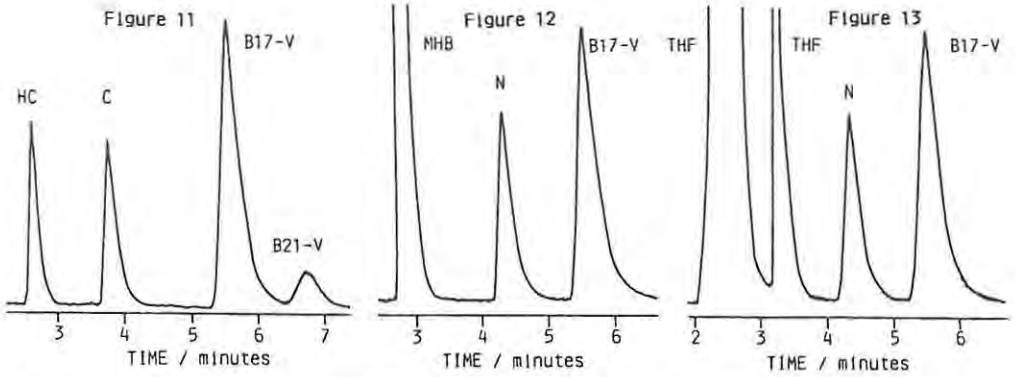
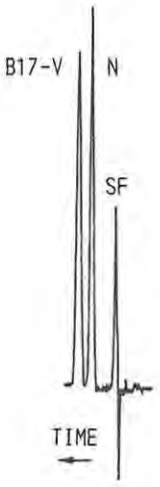


FIGURE 11-13: Typical chromatograms of a betamethasone 17-valerate cream, lotion and ointment samples. B17-V betamethasone 17-valerate, B21-V betamethasone 21-valerate, C chlorocresol, HC hydrocortisone, MHB methyl hydroxy benzoate, N norethisterone, THF tetrahydrofuran.

Specificity Studies

To ensure that formulation ingredients did not contribute to the betamethasone 17-valerate sample peak, a UV spectrum was recorded at half second intervals as the corticosteroid eluted from the analytical column after sample aliquot injection. This spectrum was compared to one obtained after injection of a pure solution of betamethasone 17-valerate. No obvious differences were noted implying that the steroidal peak obtained from the formulation sample was not augmented by other formulation ingredients, that is, there were no overlapping peaks. Additional UV spectra were similarly obtained for all other components of interest to confirm peak homogeneity. Three dimensional spectra were also obtained using the diode array detector which again confirmed the homogeneous character of each eluting peak. These spectra are shown in Figures 15-18. This high-performance liquid chromatographic method, therefore, enables specific resolution of betamethasone 17-valerate from betamethasone 21-valerate, all formulation coingredients and added internal standards.



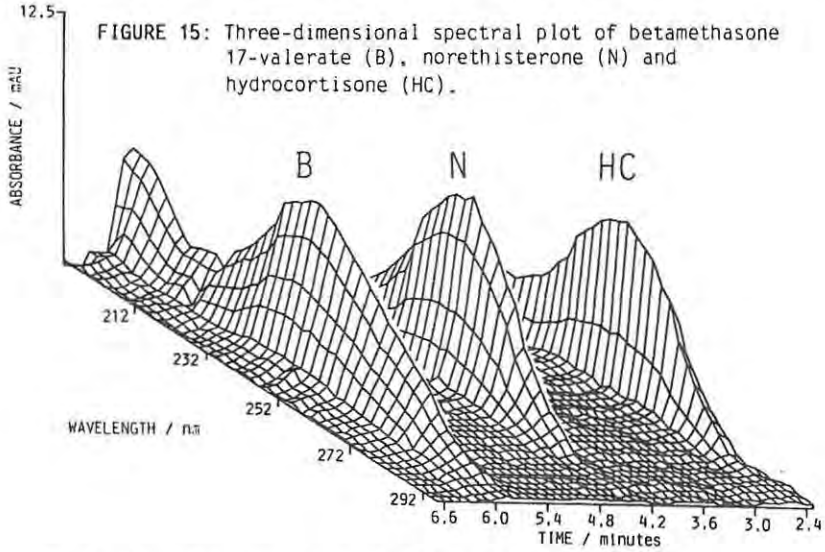


FIGURE 15: Three-dimensional spectral plot of betamethasone 17-valerate (B), norethisterone (N) and hydrocortisone (HC).

FIGURE 14: Typical chromatogrphic trace of a betamethasone 17-valerate scalp application sample. B17-V betamethasone 17-valerate, N norethisterone, SF alcoholic solvent front.

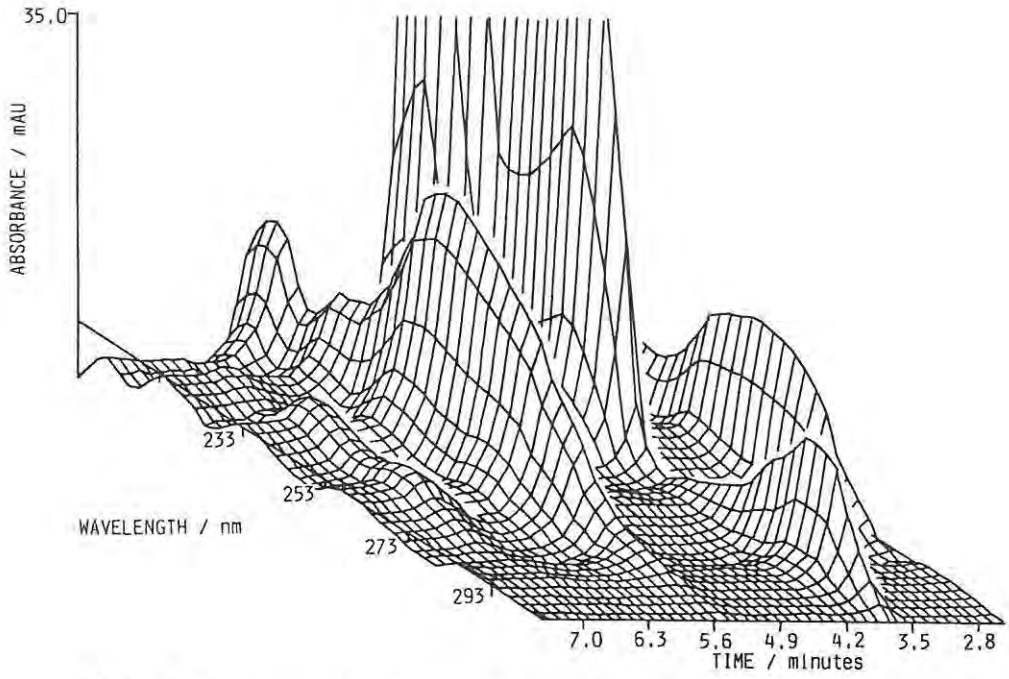


FIGURE 16: Three-dimensional spectral plot of eluents in a typical betamethasone 17-valerate cream sample.

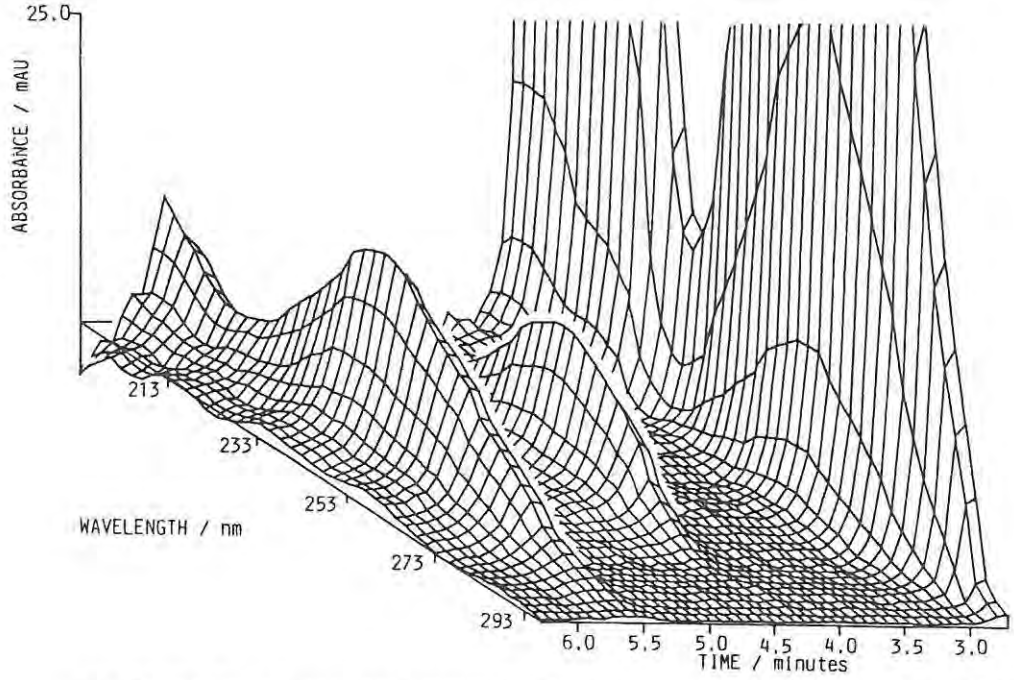


FIGURE 17: Three-dimensional spectral plot of eluents in a typical betamethasone 17-valerate lotion sample.

Linearity

Calibration curves constructed on the basis of peak height ratios of betamethasone 17-valerate/internal standard *versus* betamethasone 17-valerate concentrations were linear over the concentration range studied (2.5-20.0 μg ml^{-1}). Similar results were obtained for the betamethasone 21-valerate calibration curve over the concentration range 0.5-2.1 μg ml^{-1} (Table III).

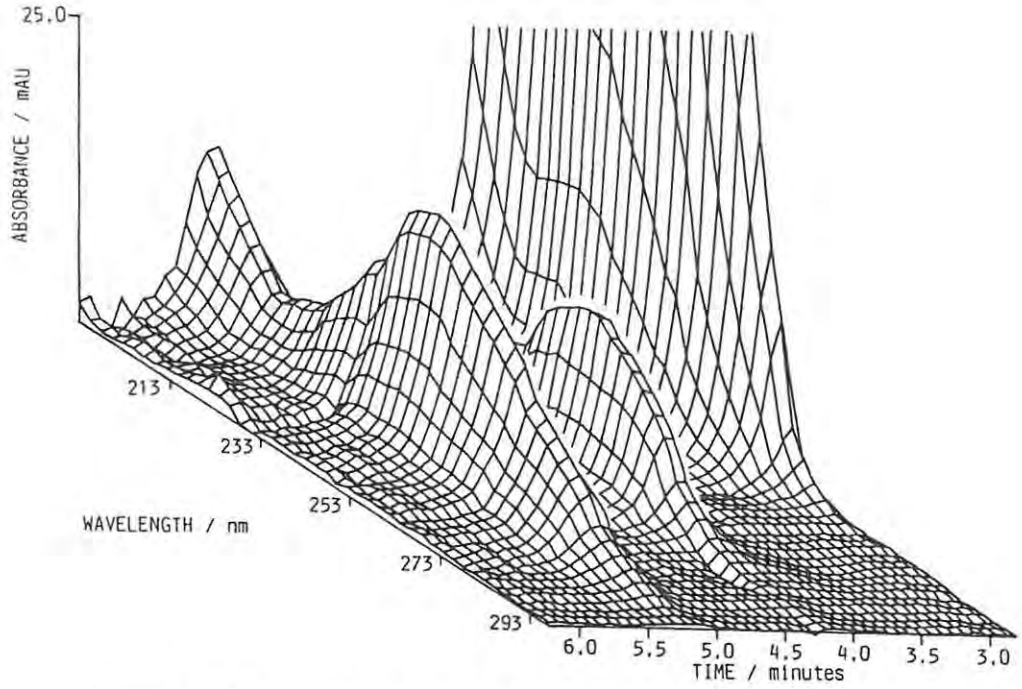


FIGURE 18: Three-dimensional spectral plot of eluents in a typical betamethasone 17-valerate ointment sample.

The linearity of these results indicate that the assay method is suitable for quantitative purposes and is sensitive to trace amounts of betamethasone 17-valerate and its degradation product.

TABLE III: Linearity data and calibration curve construction.

Compound	Internal Standard	Slope x1000	Intercept x100	Correlation Coefficient	n
B 17-V	HC	8.951	1.058	0.9998	21
B 17-V	NOR	10.518	1.844	0.9998	30
B 21-V	HC	5.381	1.164	0.9942	16

B 17-V betamethasone 17-valerate, B 21-V betamethasone 21-valerate, HC hydrocortisone, NOR norethisterone.

Precision And Accuracy

The average percentage purities of six commercial samples of each formulation type were assayed to be: cream 95.01% (SD = 0.77), lotion 99.26% (SD = 2.45), ointment 102.77% (SD = 1.73) and topical application 121.98% (SD = 3.25). There was evidence of wide variability in the lotion assay results in the absence of degradation product. This might suggest that a degree of adsorption of drug was occuring on to the walls of the plastic commercial container. Variance in results was especially marked when the samples were withdrawn from partially empty containers in which case the formulation contacting the container walls may have been dislodged on shaking. Assay values for the topical application are fairly high which is presumably due to loss of the volatile, ethanolic, base constituent either at manufacture, on

storage, or during the sampling process. Steps were taken to minimize loss during the latter manipulations and various modifications of the weighing technique were investigated, including sampling into a container fully saturated with ethanol vapour which would minimize evaporation of the vehicle base. These diverse methods yielded no significant variance in the assay value obtained. Hence, it may be postulated that the volatile component is lost from the dosage form, either at the manufacture/filling stage, or by slowly diffusing through the plastic container walls or via the closure during the several months of storage between manufacture and assay. This is assuming that no overage has been added by the manufacturer, which is unlikely.

Recovery Studies

To determine the accuracy of the method, samples of creams, lotions and ointments were initially assayed to determine the mass of betamethasone 17-valerate present in the dosage forms. Formulation samples of the same batch were then spiked with known amounts of betamethasone 17-valerate and the spiked samples were subjected to the normal assay procedure. Negligible loss of betamethasone 17-valerate is experienced during the sample preparation (Table IV), by adsorption on to lipophilic material for example, and the method is, therefore, sufficiently accurate for analytical purposes.

TABLE IV: Recoveries of betamethasone 17-valerate from spiked commercial samples.

Formulation	µg Added	µg Found	% Recovery
Creams	188.10	188.57	100.25
	187.35	189.09	100.93
	189.54	191.08	100.86
	181.87	181.20	99.63
	180.58	178.79	99.01
			Mean 100.14%
	SD = 1.218%; 95% Confidence limit = 1.514%		
Lotion	187.35	196.10	104.67
	189.45	191.55	101.11
	181.87	180.00	98.97
	188.10	187.32	99.58
	180.58	180.02	99.69
			Mean 100.80%
	SD = 2.056%; 95% Confidence limit = 2.557%		
Ointment	188.10	193.11	102.66
	187.35	193.47	103.26
	189.45	186.35	98.36
	181.87	180.33	99.15
	180.58	179.04	99.15
			Mean 100.52%
	SD = 2.264%; 95% Confidence limit = 2.814%		

On-Line Clean-Up

The column switching technique was found to be highly suitable for the clean-up of topical corticosteroid formulations thus obviating the need for

time-consuming sample preparation. Confirming reports in the literature *(257,258)*, formulation constituents did not elute from the loop column and, therefore, did not interfere with the analysis. Moreover, no deterioration of analytical column performance was experienced even after several months of daily use.

CONCLUSIONS

The recovery, linearity and specificity data indicate that this method is suitable for the rapid and simple on-line clean-up and analysis of betamethasone 17-valerate from complex topical formulation matrices without the need for conventional extractions. This method requires very small formulation sample sizes (250 mg-portions instead of several grammes), and is frugal in both analysis time and cost as the manipulative liquid-liquid extractions are avoided. The developed methodology has been found useful for diverse vehicle consistencies and viscosities. It is applicable in the presence of adjuvents such as the common topical vehicle preservatives and antibiotics such as neomycin or clioquinol. With slight modification the method is also useful for the assay of products containing hydrocortisone or fluocinolone acetonide. The method is equally suitable for the quantitative analysis of the preservatives contained within these topical formulations. Its broad spectrum applicability makes this assay extremely valuable in the field of topical corticosteroid absorption research. Although all injections and column switching were carried out manually, the potential for fully automating these procedures has already been well established *(264,258)*. The application of this automated technology would further relieve the tedium involved in day-long injection and valve switching.

3.2 RECEPTOR PHASE ASSAY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Of utmost importance in the planning and design of an *in vitro* diffusion cell system is the ability to precisely and accurately measure the quantity of permeant which passes through the membrane and enters the receptor chamber fluid. The validity of the designed system, and the interpretation of the results obtained from it, will depend exclusively on these flux measurements. With inferior concentration determination techniques, a carefully planned and designed cell system may appear to perform poorly or the variance in results

observed may suggest rejection of the model. If it can be assured that the concentration of permeant is being accurately and precisely monitored then efforts may be concentrated on improving cell design and experimental protocol, to obtain results of even greater validity. Initial planning of the diffusion cell system must, therefore, include the development of a reliable assay technique for the permeant in the receptor phase to be used.

BACKGROUND

Aqueous donor and receptor chamber fluids have been used in most of the diffusion cell system experiments which appear in the literature . This makes solute detection and quantitative analysis fairly facile by a number of techniques. In many instances radiolabelled diffusants have been used and their accumulation in the receptor fluid monitored by scintillation counting methods. This is an especially useful mode of assay as no chromatographic separation of permeants is required and operator handling is minimized. Immediate assay of the solution after each sample has been taken is also possible, even for runs lasting several days. This is seldom possible when techniques such as high-performance liquid chromatography are used as long equilibration and calibration times are required for these chromatographs which precludes start-up for a single sample injection. If these latter analyses are employed, samples must be stored until sufficient have accumulated for a full day of chromatography, for example, and this gives rise to the possible problems of permeant degradation in the sample solution.

Scintillation counting would therefore appear to be the most useful analysis technique to adopt as it allows rapid assessment without operator intensive manipulations. However, while this method may be applicable to permeation experiments using donor vehicles which have been extemporaneously prepared by incorporating radiolabelled corticosteroids, it is of no use when commercial topical products are to be tested in the *in vitro* system. The permeation of nonlabelled steroid from these proprietary products must therefore be monitored by other means.

Furthermore, a number of studies have demonstrated that aqueous vehicle phases in the diffusion cells may not present the optimum partitioning environment for diffusing molecules. In these cases permeation may be impaired by limited solubility of the diffusant in the receptor medium. Several attempts have, therefore, been made to improve the partitioning potential of these systems and, especially in the case of corticosteroids, a more lipophilic environment appears highly beneficial in this regard.

Isopropyl myristate has been used extensively as a donor and receptor

medium because of its bipolar nature and predominantly lipophilic properties which tend to mimic the biochemical composition of the skin. Isopropyl myristate was chosen for this particular study not only for these characteristics but also because betamethasone 17-valerate and betamethasone 21-valerate are highly soluble in this solvent. Moreover, being totally non-aqueous, this medium does not support the C-17 to C-21 ester rearrangement reactions which characterize the degradative transformation of betamethasone 17-valerate in aqueous environments. Solutions of betamethasone 17-valerate in isopropyl myristate are therefore stable for prolonged periods, even at room temperature. This solution stability is of benefit not only in preserving the character of the permeant once it has entered the receptor chamber fluid but also in retaining its integrity once a sample has been removed from the cell and is in storage, prior to analysis.

It was therefore necessary to develop a method of analysis for betamethasone 17-valerate and betamethasone 21-valerate in isopropyl myristate solution. In some reported cases the receptor fluid has been channeled directly to a spectrophotometer by peristaltic pump for continuous detection of UV absorbance as the permeation experiment proceeds *(64,208)*, or samples have been removed from the receptor compartment and assayed directly by spectrophotometer *(84)*, in some cases after reaction with acid hydrazide reagent *(223)*. Personal experience has demonstrated this method to be unsuitable because there may be a number of permeating compounds that enter the receptor fluid and contribute to the measured absorbance, thereby overestimating the actual quantity of drug present in the solution. This problem is well exemplified by monitoring the permeation of compounds from commercial betamethasone 17-valerate creams into hexane receptor phases. This corticosteroid is sparingly soluble in hexane and therefore its appearance in the receptor phase is minimal over a period of time. Conversely, the cream preservative, chlorocresol, is highly soluble in hexane and its concentration increases rapidly in this receptor solvent. By direct spectrophotometric measurement the absorption due to the preservative is indistinguishable from that due to the drug and erroneous conclusions may therefore be made. Similarly, methodology has been reported by Waranis *et al. (88)* where prolonged periods of receptor phase contact with diffusion cell membrane have been practiced prior to the commencement of the diffusion experiment to exstract any UV absorbing materials from the tissue which may interfere with direct spectrophotometric detection. Analytical separation of the receptor phase constituents would obviate the necessity for this procedure and would result in more accurate measurement of the permeant. The deleterious effects of prolonged skin contact with the solvent is, thereby, also avoided.

Chromatography of the receptor chamber solution is therefore imperative to distinguish between permeant, degradation products and preservatives. Poulsen *et al. (14)* have used thin layer chromatography to separate radio-labelled corticosteroids in isopropyl myristate; although this method qualitatively confirmed that measured radioactivity was due to permeation of the parent compound, accurate quantitative assessment is more difficult. High-performance liquid chromatography appears to be the analytical technique of choice for the separation and analysis of permeants in diffusion cell receptor phases, although this methodology has been adopted by relatively few researchers *(81)*.

Therefore, a high-performance liquid chromatographic method was developed to accomplish the separation and stability-indicating quantitative analysis of betamethasone 17-valerate in isopropyl myristate. The method is very similar to that described previously for the assay of topical formulations and, again, uses a column-switching technique for the isolation of the drug moieties from the lipid phase. In this case the lipid matrix presented by the isopropyl myristate is not nearly as complex as that of the topical formulations, but still poses significant chromatographic problems. With slight modification the method is equally applicable to measurement of hydrocortisone flux rates.

MATERIALS AND METHODS

These details are essentially similar to those described previously for the analysis of topical dosage forms (Section 3.1) and therefore only brief summaries are given here. Differences to the previous description in the materials or methods used are elaborated upon.

Reagents And Chemicals

The sources of the acetonitrile, methanol, chromatographic grade water, betamethasone 17-valerate, betamethasone 21-valerate and norethisterone were described previously. These chemicals were spectral or analytical reagent grade and were used as received. The isopropyl myristate was rated 98% pure, synthesis grade (Merck, West Germany); samples of higher purity were not available commercially. The propylene glycol (Saarchem, South Africa) was rated 95% pure, synthesis grade.

Apparatus

The high-performance liquid chromatograph consisted of a syringe loading, six-port sample injector equipped with a loop column, an analytical column coupled to either a strip chart recorder or an integrator, all as described previously. In this case the system consisted of a single solvent delivery system (Model 6000A, Waters, U.S.A.), a variable wavelength UV detector (Model SF769, Kratos Analytical Instruments, U.S.A.) and loop column flushing was accomplished manually by syringe.

HPLC Conditions

The mobile phase composition used was the same as described previously and methods of preparation, filtering and degassing were identical. The mobile phase flow rate was set at 1.5 ml min^{-1} and all chromatography was carried out at ambient temperature. The detector wavelength used was 239 nm.

Purification Of Isopropyl Myristate

To assess its purity, an aliquot of the isopropyl myristate was extracted with an equal volume of acetonitrile and 10 µl of the latter injected into the chromatograph. A large absorption band and a smaller peak were noted which eluted between approximately 4.5 and 6.5 minutes, presumably due to an impurity contained in the isopropyl myristate. An extraction of the lipid phase with acetonitrile containing betamethasone 17-valerate and norethisterone in solution (Figure 19) indicated that, while it may be possible to resolve the norethisterone peak from the large impurity band, betamethasone 17-valerate eluted between the two impurity peaks making resolution impossible. Spectral analysis of the impurity peaks at 4.95 and 5.88 minutes (Figure 20) indicate

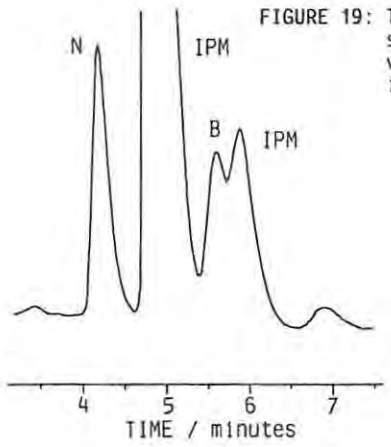


FIGURE 19: Typical chromatogram of an unpurified isopropyl myristate extract showing elution interference of the impurities with betamethasone 17-valerate peak. B betamethasone 17-valerate, IPM impurities in isopropyl myristate, N norethisterone.

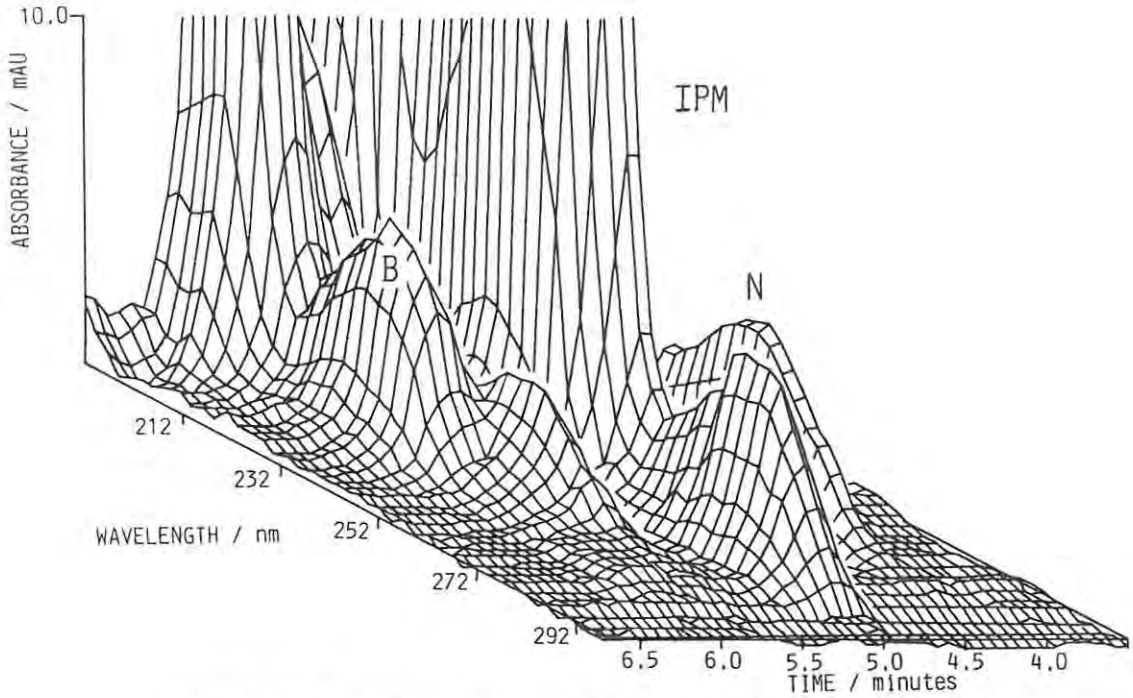


FIGURE 20: Three-dimensional spectral plot of betamethasone 17-valerate (B), impurities in isopropyl myristate (IPM) and norethisterone (N).

that both peaks have a UV absorbance maximum at approximately 225 nm implying that these peaks are probably not due to impurities of steroidal origin. Similar spectral analysis of the betamethasone 17-valerate and norethisterone peaks demonstrate the indicative steroidal absorbance maximum at 240 nm. Purification of the commercial isopropyl myristate was therefore essential if this chromatographic technique was to be used for the analysis of the receptor chamber solution.

Successive extractions with acetonitrile diminished the peak heights of the impurities, however the acetonitrile also solubilized some of the isopropyl myristate at each extraction. The purification of relatively large volumes of the lipid with this extractant was therefore not feasible. Extractions using purified water had no effect on the magnitude of the impurity peaks, the solutes were thus highly water insoluble. A search was therefore instigated for liquids of high solvent potential which were immiscible with isopropyl myristate. Propylene glycol appeared to suit these requirements perfectly. Successive extractions of the lipid phase with aliquots of propylene glycol significantly diminished the recorded absorbance due to the impurities. Total removal of the impurity was effected by 15 isovolumetric extractions. The subsequent absence of impurity in the isopropyl myristate was demonstrated by acetonitrile extraction and injection of the extractant.

This method was adopted for purification of isopropyl myristate used in all subsequent diffusion experiments. Large volume separating flasks were used for the partitioning process with centrifugation of the upper, lipophilic

layer following the final propylene glycol extraction. With this preliminary clean-up step of the receptor chamber fluid the high-performance liquid chromatographic technique could be optimized for the analysis of permeant corticosteroids present in solution without the interference of commercial impurities.

Internal Standard Solutions

Under the operating conditions described above, it was found that the high-performance liquid chromatography system adequately resolved the norethisterone internal standard, (4.2 min), from betamethasone 17-valerate (5.6 min), betamethasone 21-valerate (6.5 min) as well as the chlorocresol (3.6 min) and methyl hydroxy benzoate (2.9 min) preservatives, for quantitative purposes. Resolution of the internal standard peak from the ethanol-enhanced solvent front due to permeation from the scalp application dosage form was also achieved. Internal standard solution was prepared in acetonitrile/water (55/45) mobile phase at a concentration of 3.5 $\mu g\ ml^{-1}$ norethisterone.

Calibration Standard Solutions

Standard stock solutions of betamethasone 17-valerate were prepared in purified isopropyl myristate at concentrations of 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0 and 40.0 $\mu g\ ml^{-1}$. Each solution was used to test for linearity of detector response and recovery characteristics of the steroid from the isopropyl myristate. Similarly, in order to quantitate the degradation product, betamethasone 21-valerate solutions were prepared in isopropyl myristate at concentrations of 0.5, 1.0, 1.5 and 2.1 $\mu g\ ml^{-1}$.

A larger range of betamethasone 17-valerate mass was tested in this assay, compared to the formulation assay, to account for the magnitude of the permeant expected to appear in the receptor cell solution over several days of experimentation. In a typical diffusion run the receptor isopropyl myristate would be devoid of drug at the start of the experiment and permeant concentration would increase gradually with time. Therefore, in essence, this analytical procedure would have to accurately measure the drug over a wide concentration range, whereas the formulation assay simply measured the variance in concentration in the vicinity of the label claim datum. Linearity of detector response and recovery from the lipophilic matrix over a wide concentration range were therefore vital in the applicability of this method.

Sample Preparation

Isopropyl myristate samples of 40 µl were withdrawn from the receptor chamber of the diffusion cell at specified time intervals using a fixed volume transfer pipette (Brand, West Germany). These samples were placed into small, glass collection tubes (4 mm i.d. x 50 mm long), the tubes were covered (Parafilm, American Can Co., U.S.A.) and stored under refrigeration (4 °C) until assayed. Prior to injection, 40 µl of internal standard solution containing 3.5 µg ml^{-1} norethisterone was added to the isopropyl myristate sample in each tube and the mixture was vortexed for 30 seconds ensuring a homogeneous, fine dispersion of the two immiscible phases. The samples were then centrifuged for 60 seconds to assist in complete separation of the phases and 10 µl of the lower, mobile phase layer was withdrawn by syringe ready for injection into the chromatograph. This simple liquid-liquid extraction step would induce partitioning of the betamethasone 17-valerate and norethisterone between the chamber isopropyl myristate and the internal standard solution.

Standard solutions were prepared for linearity studies in a similar fashion. Aliquots of calibration standard solution (40 µl) were added to 40 µl portions of internal standard solution in sample tubes. These mixtures were vortexed and centrifuged as described previously and 10 µl of the lower layer removed for injection. In this manner the partitioning characteristics of the corticosteroids between the isopropyl myristate and internal standard solution could be investigated over the concentration range studied.

Injection Procedure

A 10 µl volume of sample or calibration standard solution was injected into the valve placed in the "load" position (off-line). Without removing the syringe the injector valve was turned to the "inject" position (on-line), thus introducing the injection aliquot into the chromatographic system. The syringe was then withdrawn and the valve left in the inject position for two minutes to allow only the components of interest to pass through the loop column and transfer to the analytical column. The injector was then turned back to the load position. This procedure trapped any lipophilic fraction which had dissolved in the internal standard solution, during the liquid-liquid extraction process. This lipid component was then manually forward-flushed off the loop column by syringe using 1 ml of methanol followed by 5 ml of mobile phase. In this manner no re-equilibration of the loop column with the remainder of the analytical system was required prior to the next

injection. It was found that methanol aliquots were adequate for displacing bound lipophilic components from the loop column, probably due to preferential hydrogen bonding capacity with the packing silanol groups as discussed previously.

Calibration standard solution injections were interspaced between the sample injections, ensuring that each solution was injected at least three times for means to be calculated. Peak height ratios of injected samples could be compared with ratios from calibration standard solutions in order to assign a value to the mass of corticosteroid present in the isopropyl myristate aliquot.

Calculations

All determinations of corticosteroid content from the resultant chromatograms were carried out using the relative response factor method as detailed previously.

RESULTS

Figure 21 depicts chromatographic traces of the isopropyl myristate receptor chamber solutions obtained from diffusion runs using different topical betamethasone 17-valerate commercial formulations (Betnovate, Glaxo, South Africa) and human stratum corneum as the diffusion medium. A typical chromatogram of isopropyl myristate containing betamethasone 17-valerate from a cream diffusion experiment is shown in Figure 21a.

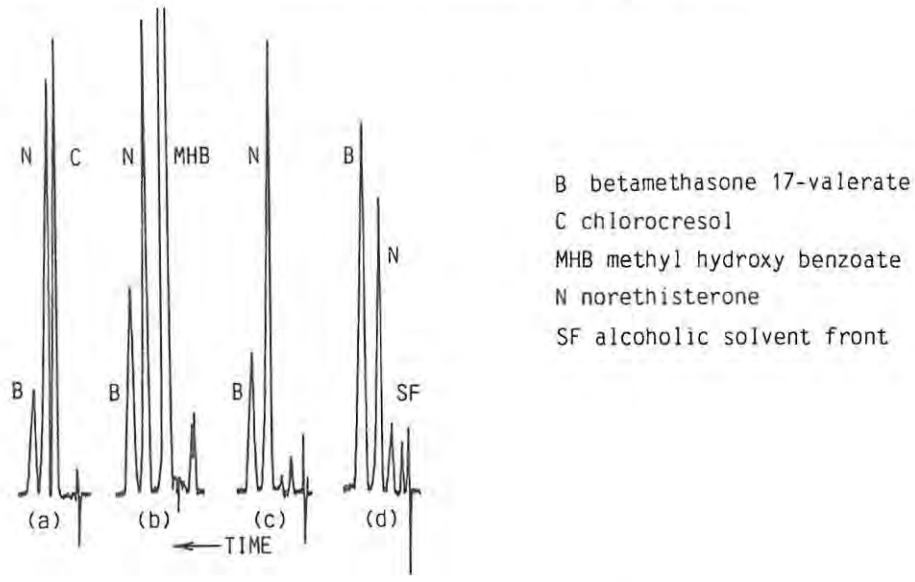


FIGURE 21: Typical chromatographic traces from extracts of isopropyl myristate receptor phases from cream (a), lotion (b), ointment (c) and scalp application (d) permeation experiments.

This trace depicts the resolution between the preservative (chlorocresol) and the internal standard (norethisterone). Figure 21b is a chromatogram from a typical lotion diffusion run, including the internal standard and demonstrates the large absorption peak produced by the methyl hydroxy benzoate preservative. Figure 21c shows the relatively simple chromatogram from an ointment run, in this case without preservative peak, and the chromatographic trace from a topical application diffusion experiment is shown in Figure 21d.

Specificity Studies

To ensure that isopropyl myristate fractions or components which may have leached from the diffusion medium did not contribute to the betamethasone 17-valerate sample peak, a UV spectrum was recorded at half second intervals as the corticosteroid eluted from the analytical column after sample aliquot injection. This spectrum was compared to one obtained after injection of a pure solution of betamethasone 17-valerate. No obvious differences were noted implying that the steroidal peak obtained from the lipid receptor solution was not augmented by any other constituents present. Additional UV spectra were similarly obtained for the internal standard to confirm peak homogeneity.

This high-performance liquid chromatographic method, therefore, enables specific resolution of betamethasone 17-valerate from betamethasone 21-valerate, all lipid fraction ingredients and added internal standard.

Linearity

Calibration curves constructed on the basis of peak height ratios of betamethasone 17-valerate/internal standard *versus* betamethasone 17-valerate mass were linear over the concentration range studied (2.5-40.0 $\mu g\ ml^{-1}$). Similar results were obtained for the betamethasone 21-valerate calibration curve over the concentration range 0.5-2.1 $\mu g\ ml^{-1}$ (Table V).

TABLE V: Linearity data and calibration curve construction.

Compound	Internal Standard	Slope x1000	Intercept x100	Correlation Coefficient	n
B 17-V	NOR	2.680	1.343	0.9992	30
B 21-V	NOR	1.371	0.295	0.9958	16

B 17-V betamethasone 17-valerate, B 21-V betamethasone 21-valerate, NOR norethisterone.

The linearity of these results indicates that the assay method is suitable for quantitative purposes over a wide betamethasone 17-valerate concentration range and is sensitive to trace amounts of the investigated corticosteroids.

Precision, Accuracy And Recovery Studies

To determine the precision, accuracy and recovery of the method, solutions of betamethasone 17-valerate were made up in the normal internal standard solution at concentrations of 5.0, 10.0, 20.0, 30.0 and 40.0 μg ml^{-1}. Aliquots of these solutions (40 μl) in acetonitrile/water were added to 40 μl portions of purified isopropyl myristate, vortexed and centrifuged as previously described. Samples from the lower separated layer were injected into the chromatograph and the resultant peak height values compared to the calibration curve values at equivalent betamethasone 17-valerate concentration.

The calibration curve was constructed from solutions of betamethasone 17-valerate solubilized in isopropyl myristate, to which internal standard aliquots were added. In this study the corticosteroid was solubilized in the internal standard solution and aliquots of this mixture were added to drug-free isopropyl myristate. In essence, the degree of partitioning of the betamethasone 17-valerate from and into the lipid phase was compared. Obvious variance in the peak height values observed in this mode of comparison would indicate anomalies in the partitioning of betamethasone 17-valerate between the isopropyl myristate and internal standard solution. This comparison would also give some indication of the precision and accuracy of the analytical procedure.

The results in Table VI confirm equivalent degrees of partitioning into and from the isopropyl myristate, as expected, and that negligible loss of betamethasone 17-valerate is experienced during the sample preparation. The method is, therefore, sufficiently accurate and precise for analytical purposes.

TABLE VI: Recoveries of betamethasone 17-valerate from spiked isopropyl myristate samples.

μg Added	μg Found *	% Recovery		
50.00	49.62	99.24		
	50.80	101.60	Mean	99.90
	48.80	97.59	SD	1.85
	50.59	101.18		
100.00	98.64	98.64		
	100.40	100.40	Mean	99.04
	99.22	99.22	SD	1.06
	97.88	97.89		
200.00	199.30	99.65		
	195.70	97.85	Mean	99.71
	203.90	101.95	SD	1.69
	198.77	99.38		
300.00	304.38	101.46		
	296.28	98.76	Mean	100.37
	292.45	97.48	SD	2.85
	311.62	103.87		
400.00	397.84	99.46		
	394.36	98.59	Mean	99.84
	403.63	100.91	SD	1.02
	401.53	100.38		

* as assessed by reference to calibration curve.

On-Line Clean-Up

The column switching technique was found to be highly suitable for the clean-up of acetonitrile/water solutions used to accomplish liquid-liquid extractions of isopropyl myristate. The primary extraction step separated the bulk of the lipid medium, however a small portion of lipophilic material was dissolved in the extractant which was injected into the chromatographic system. Without further clean-up, this material causes a broad elution band at approximately 12 minutes and the system requires at least 10 further minutes for base line re-equilibration. This dictates the adoption of further extraction/ clean-up procedures for the extractant solution, a prescription which is efficiently accomplished by multicolumn chromatography. The column-switching technique was able to totally isolate this lipophilic fraction from the analytical system and, thereby, no interference with the detection of the corticosteroids nor system equilibrium resulted. Furthermore, by conducting this loop column sequestration of the lipophilic material no deterioration of analytical column performance was experienced even after a year of continuous use.

CONCLUSIONS

The high-performance liquid chromatographic technique thus developed was demonstrated to be highly efficient in the analysis of betamethasone 17-valerate concentration in isopropyl myristate used as the receptor phase of *in vitro* diffusion cells. Extraction of the drug moiety from the lipid phase was effected by a combination of liquid-liquid extraction and on-line sample clean-up using a switching valve and loop column assembly. The method is shown to be relatively simple, accurate and linear over the broad permeant concentration range experienced in diffusion experiments and has the capability of quantifying degradation products in the cell receptor solution. The major advantage of having this reliable analytical technique available is that it allows the accurate monitoring of nonradiolabelled corticosteroid diffusion from commercial formulations. Slight modifications to the methodology have allowed the assay of other corticosteroids, such as hydrocortisone, in the receptor fluid indicating that the method is highly applicable to a diverse range of membrane diffusion experiments.

3.3 METHODS OF MEMBRANE PREPARATION

The techniques and chemicals used in the pretreatment and preparation of membranes may have a profound effect on the flux rates of drugs which diffuse through them. The theory of lipid extraction by organic solvents contacting skin samples and the deleterious effects that proteolytic enzymes may have on the barrier layer have already been discussed. Theoretically, the use of chemicals or conditions which may alter the barrier characteristics of the medium should be avoided wherever possible. Cleaning and handling are more likely to induce trauma in biological tissue than in the relatively inert synthetic materials. Additional care must, therefore, be exercised in the isolation of delicate samples of skin in comparison to the sturdier, manufactured polymers. Of equal importance is the adherence to uniform preparative techniques for all samples of the same material so that reliable conclusions may be drawn from the diffusion results. The actual manipulations may vary from simple rinsing of purchased synthetic membrane in water to the elaborate means of removing intact tissue samples from living donors with the minimum of trauma and scarring. All methods require a definite experimental protocol to be developed, evaluated and perfected, dependent on the source and complexity of the sample.

SYNTHETIC MEDIA

Cellulose Membrane

Cellulose dialysis tubing (Visking, Serva, West Germany) rated at a molecular pore cut-off of 8000-15000 daltons for dialysis, average pore diameter 4.8×10^{-9} m, was used in the diffusion experiments. This regenerated cellulose contains various plastisizer and preservative additives (glycerol for example) which are usually UV absorbing materials that may leach into the receptor chamber solution and interfere with chromatographic analysis. The removal of these additives from the membrane is therefore imperative and, being generally water soluble compounds, they may be washed away by soaking or, as recommended by the manufacturer, boiling the membrane. It is assumed the degree of additive extraction may influence the flux of diffusants passing through the membrane. If additives are only minimally removed, they may interact with the diffusant by adsorption or may occupy interpolymer pores thereby hindering the passage of other moieties.

To investigate this possibility, the dialysis membrane used in these diffusion experiments was prepared in three different ways: firstly, membrane

was soaked in purified water (high-performance liquid chromatographic grade, prepared as described in section 3.1), at 20 °C for 60 minutes. Secondly, dialysis tubing was soaked in purified water at 60 °C for 60 minutes, and, lastly, the tubing was boiled in purified water for 60 minutes. In all cases the dialysis tubing was cut into square sections of approximately 4 cm^2, immersed in about 250 ml of water and continuously agitated during the soaking process by teflon bar magnet and laboratory stirrer set at 200 rpm. After soaking the membrane cylinder was cut open forming a rectangular sheet of single layer cellulose membrane. This sheet was rinsed with fresh purified water, blotted dry using tissue paper, and mounted in the diffusion cells prior to initiating the diffusion experiment.

Filter Membranes

The porous filter membranes (see Plate 4) used in this investigation (8.0 µm Polycarbonate membrane, Nuclepore, U.S.A.) are not reported to contain any additives on purchase. Preparation of this media was, therefore, fairly facile and only involved soaking the membrane discs (47 mm in diameter) in purified water at 20 °C for 60 minutes. Thereafter, the discs were rinsed with fresh water, blotted dry, cut to size and mounted in the diffusion cells. The soaking procedure was conducted simply to maintain uniformity in membrane preparation as the filter material is reported to be nonhygroscopic and has a very low adsorptive potential. The degree of hydration which the medium undergoes during immersion is, therefore, thought to be minimal.

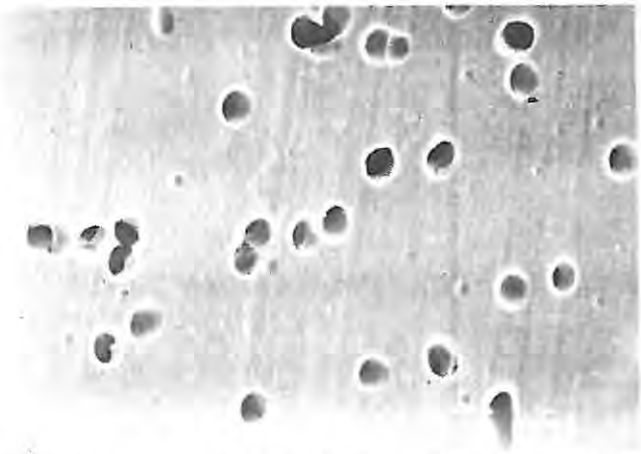


PLATE 4: Topography of porous filter membrane (Polycarbonate, Nuclepore, U.S.A.).

Silicone Membrane

The polydimethylsiloxane membrane (Silastic, type 500-1, Dow Corning, U.S.A.) used for this research did not contain filler elements nor fabric

reinforcing and was 0.127 mm thick (see Plate 5). The polymer is shipped from the manufacturer covered in a film of sodium bicarbonate powder, which facilitates handling and packing of the synthetic sheets. This dusting powder must be removed from the membrane prior to experimentation. The manufacturer suggests washing the silicone in a mild soap solution; this method was rejected on the basis that surfactant molecules may adsorb on to, or diffuse into, the membrane and may not be efficiently removed by water rinsing. The presence of surfactants may enhance solubilization, partitioning and diffusion of the relatively hydrophobic corticosteroids. It was decided to repeatedly soak and rinse the membrane in numerous water immersions which would effectively remove the water soluble, superficial powder. The whole sheet (20 x 15 cm) was immersed in approximately 500 ml of purified water and agitated for 15 minutes by bar magnetic stirrer. After this initial soaking, the membrane was rinsed with fresh water and the entire process was repeated three times. The sheet was blotted dry and stored between sheets of tissue paper until needed.

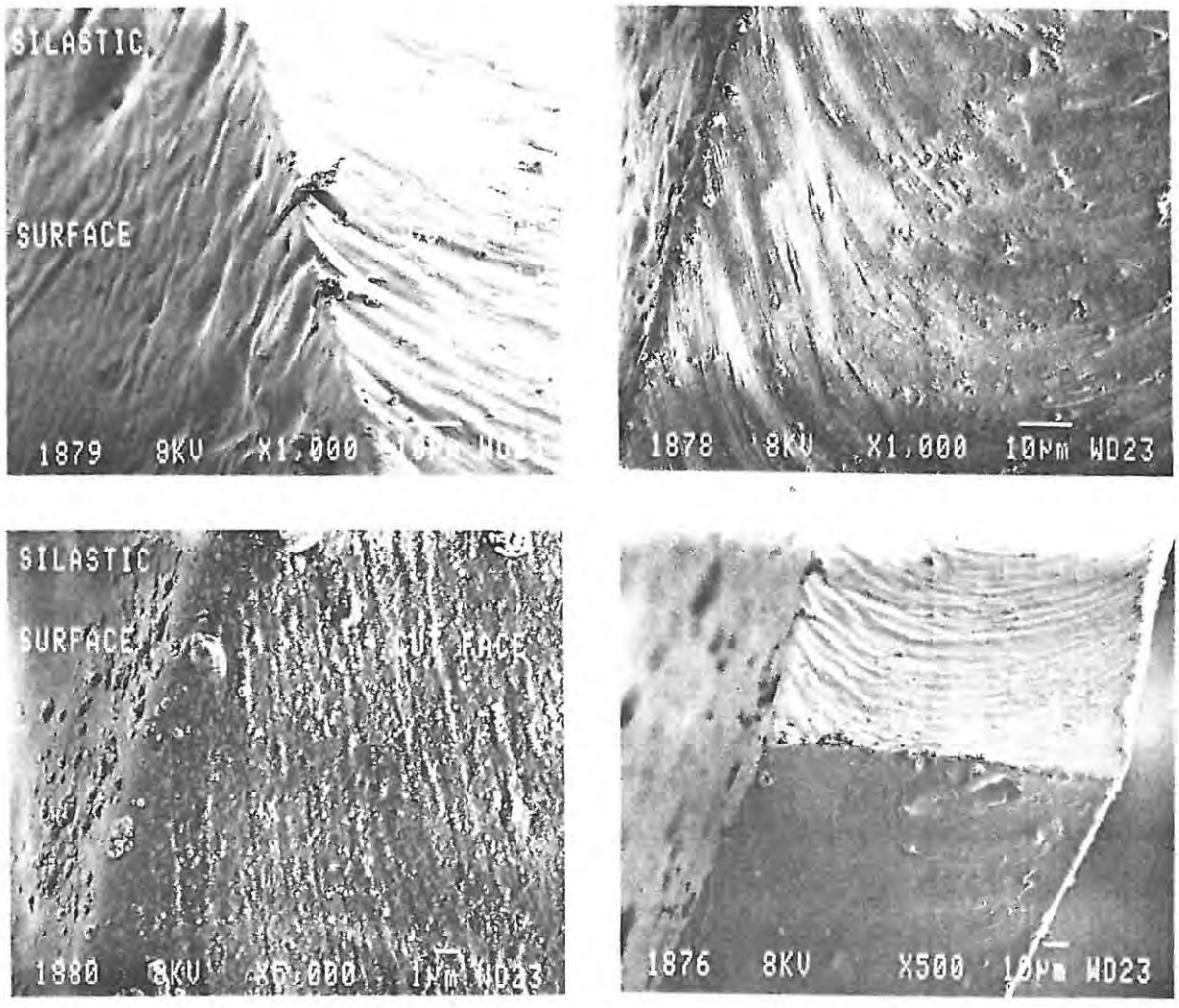


PLATE 5: Topography and transverse sections of silicone membrane (Silastic, Dow Corning, U.S.A.).

ANIMAL MEDIA

Hairless Mouse Skin

Male hairless mice of HRS/J strain (genotype *hr/hr*, Jackson Laboratory, U.S.A.) were isolated in cages at approximately one month of age to prevent fighting-inflicted injury to the skin, and resultant scar tissue formation. Animals approximately nine weeks old and weighing 25 g $\pm$ 1.8 g were sacrificed by carbon monoxide euthanasia for use in diffusion experiments. A scalpel incision was made through the skin on the ventral surface of the animal from cervix to lower abdomen with care being exercised to avoid puncturing the peritoneal cavity. Abdominal and dorsal full thickness skin was then separated from the peritoneal lining by blunt dissection, avoiding the limbs, genitalia and tail regions. The excised skin was spread on a dissection tile, dermal surface exposed, and subdermal adipose tissue, blood vessels and other debris were removed by gentle scraping with a blunt spatula. The cleaned, full thickness skin (0.7-1.0 mm thick) was then rinsed with water, blotted dry and either cut to size for mounting in a diffusion cell or spread on a sheet of plastic for refrigerated storage at -10 °C until required.

Skin which had been frozen was allowed to thaw gradually at room temperature, immersed for approximately 30 minutes in purified water at 20 °C and then blotted dry prior to cutting and mounting in the diffusion cell. The results from the experiments indicate that there was no significant difference in the permeability of the skin used immediately after sacrifice and that stored in the frozen state for up to three weeks *(17,35,147,156)*. Neither was there significant dissimilarity in the results of skin taken from the dorsal or abdominal regions, confirming the reports of Behl *et al. (29,190)*. Animal skin sampling in this manner was, therefore, frugal in tissue usage as very little membrane taken from each animal was discarded. Only male mice older than nine weeks were used in the diffusion experiments to minimize the possible age and hormonal influences on diffusion and metabolism which have been suggested by Behl *et al. (190)* and Valia and Chien *(81,116)*, respectively, to be significant factors when young or female skin is used.

Attempts to separate the epidermis and stratum corneum from the dermis of this excised skin by immersion in water at 60 °C for two minutes were unsuccessful. Confirming the problems experienced by Lee and Parlicharla *(158)*, only very small, dispersed areas of tissue could be separated in this manner which were grossly inadequate for mounting in the diffusion cell. Full thickness hairless mouse skin was, therefore, employed in all the diffusion experiments. Plate 6 shows the topographical and sectioned nature of the full

thickness skin.

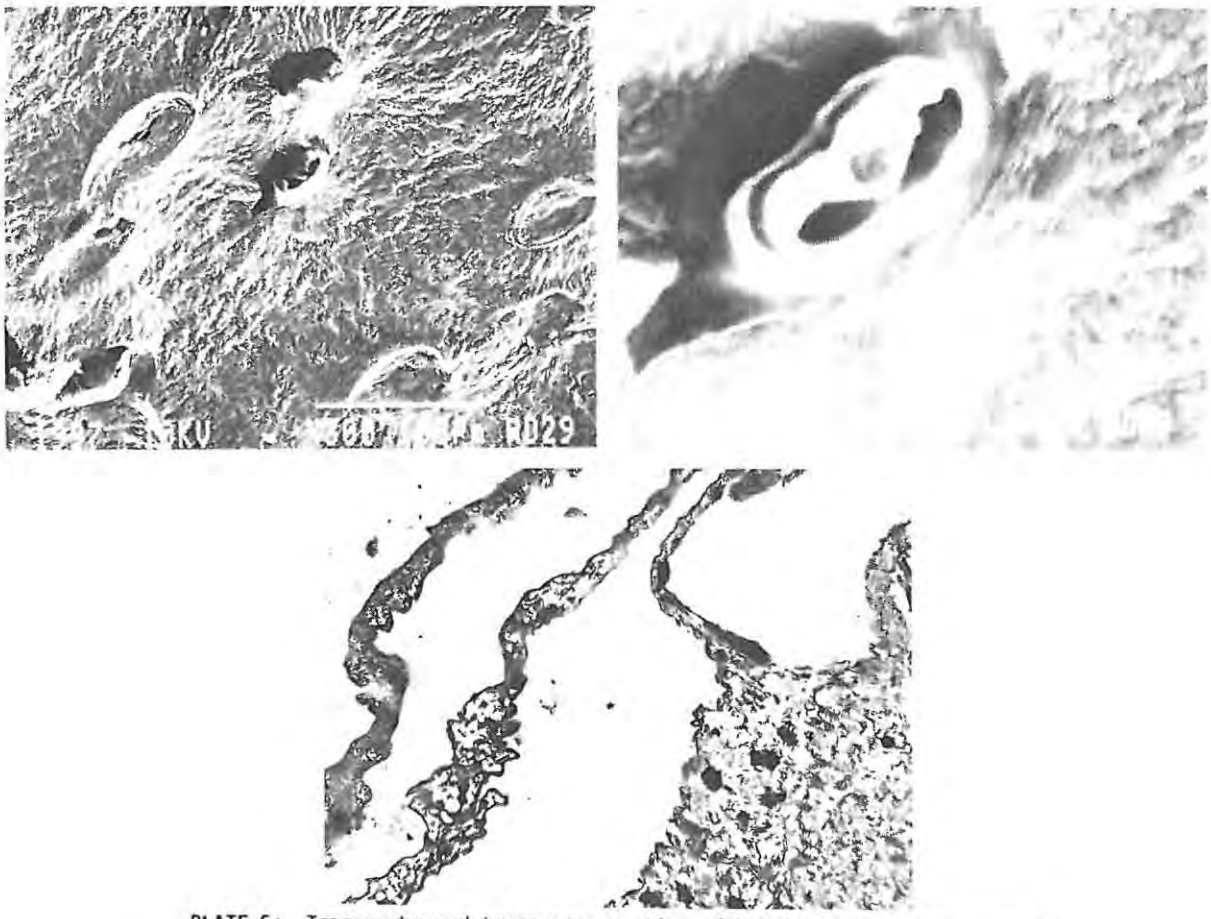


PLATE 6: Topography and transverse section of hairless mouse skin showing hair follicle and relatively thin stratum corneum.

A problem anticipated with the use of full thickness skin was the possibility of metabolism of the permeant on its passage through the tissue, or leaching of catabolic enzymes into the receptor chamber medium and subsequent catabolism of the permeants once they had partitioned into this fluid. It has been shown *(194,195)* that a large percentage of the water soluble enzymes present in the skin may be abstracted from it by simple soaking in aqueous media. This enzyme leaching predominantly occurs in the first few hours of immersion and was conveniently accomplished in this study by the tritiated water integrity testing technique applied to each biological membrane. This methodology required a purified water receptor fluid to contact the dermal surface of the skin membrane for at least six hours, sufficient time for extensive enzymatic removal. No metabolic byproducts of the permeants were detected by the stability-indicating high-performance liquid chromatographic method used to assay the receptor chamber isopropyl myristate solution. The corollary that this was expressly due to the enzyme abstraction step is neither implied nor proven. Suffice it to say that the contact of the skin with the aqueous medium for several hours prior to the start of the diffusion experiment could only be of benefit in removing some

catabolic enzymes which may have interfered with the accurate measurement of permeant mass. The immersion process would, presumably, also be of benefit in the rehydration and equilibration of the membranes which had been frozen.

Pig Skin

The abdominal tissue of male Duvoc/Landrace/Large White mixed strain weanling pigs was used in the diffusion experiments. The animals died at birth, or soon thereafter, from natural causes and the abdominal skin was excised as soon as possible post mortem. This anatomical region was chosen because it is the least hirsute on the animal, and would facilitate further separation of the skin strata. Bilateral incisions were made through the skin on either side of the abdomen extending from the forelegs to the umbilical region. Transverse incisions isolated a rectangle of tissue which was then excised from the animal corpse by dissection through the extensive subdermal adipose layer. The excised membrane was several millimetres thick, comprising mainly of adipose material.

The excised skin was placed on a dissection tile, epidermal surface exposed, and the few, coarse hairs were clipped close to the skin surface using a dissection scissors. Care was taken not to damage the horny layer in this process. The stratum corneum/epidermis was then separated from the dermis using a heat separation technique *(28,157,161)*. The clipped full thickness skin was totally immersed in 60 °C water for two minutes. Thereafter the epidermal layer could fairly easily be teased from the dermal stratum with the aid of blunt dissection. This separation was hindered in places by the relatively large hair follicles and remaining hair shafts which tended to bind the layers together. The epidermal tissue was then rinsed with purified water, blotted dry and either cut for cell mounting or spread on a plastic sheet for refrigerated storage until required. Plate 7 shows the surface and sectioned characteristics of this prepared membrane.

The effect of the rupture of large hair follicles and eccrine glands caused by this technique on the integrity of the prepared membrane was uncertain. The actual clipped hair shafts were not removed with the upper layers and remained fastened to the dermal tissue. This would suggest that a hole would remain at each point where the hair shaft passed through the epidermis and horny layer, a factor that could grossly impair barrier potential. Histologically, the stratum corneal layer is not a flat sheet but penetrates and covers the inner surfaces of the follicles and glands. On stratal separation the horny layer infundibula protrude from the epidermal surface of the membrane and tend to coalesce, diminishing the size of the

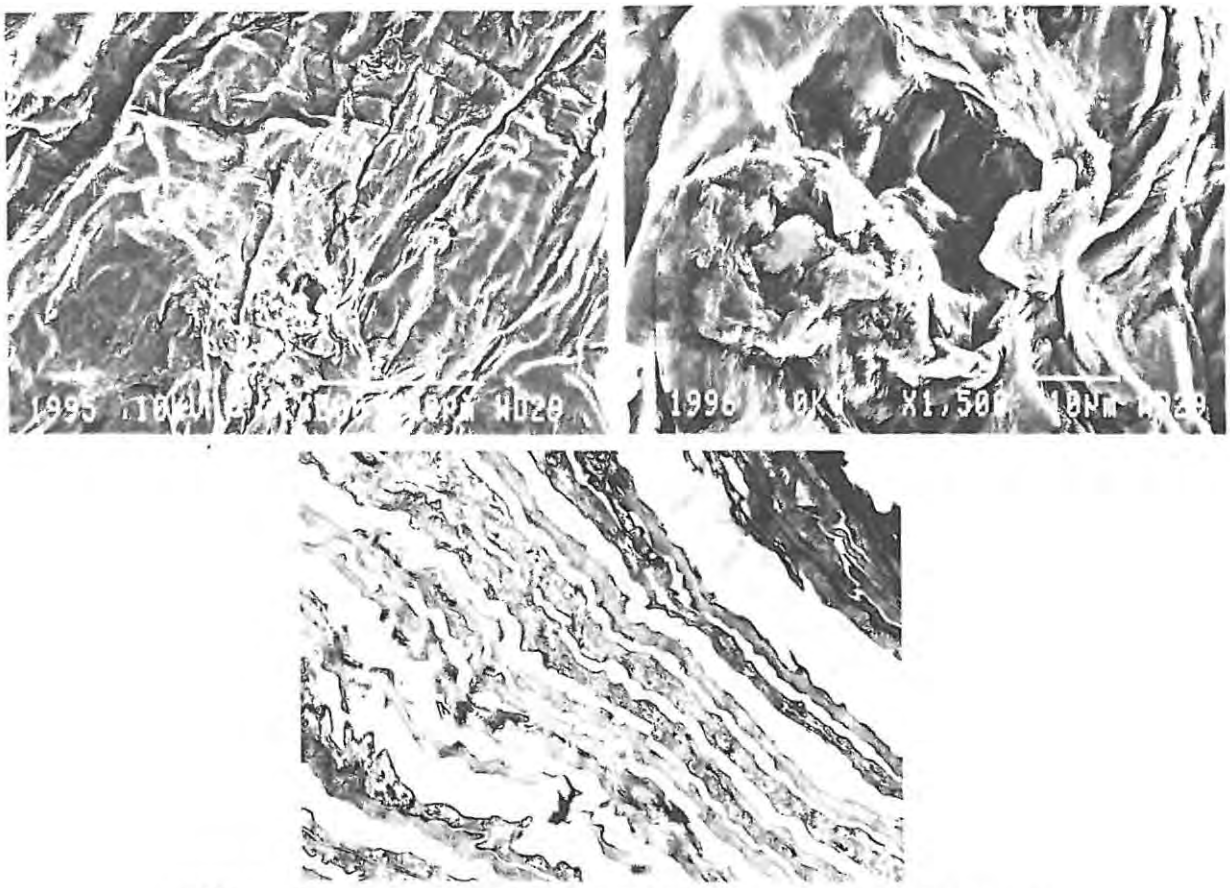


PLATE 7: Topography and transverse section of weanling pig stratum corneum showing relatively thick stratum corneum.

orifice which accomodated the hair shaft. The integrity of the membrane as a diffusive barrier is, therefore, generally preserved: unless extensive damage has been generated in the separation of the strata. Major trauma of this nature would be immediately apparent in the results of the tritiated water diffusion experiments, and the membrane in question could be rejected. The problem is especially pronounced with pig skin which has relatively large hair follicles and shafts; pores produced by rupture would represent a relatively larger membrane area than that resulting from the similar preparation of, for example, human skin. Less significant impairment of the barrier layer was, therefore, expected in the blister preparation of human stratum corneum. Nevertheless, in all instances the tritiated water permeation results would rapidly identify those membranes unsuitable for further diffusion experiments.

As with mouse skin, weanling membrane which had been frozen was allowed to thaw gradually at room temperature, immersed for approximately 30 minutes in purified water at 20 °C and then blotted dry prior to cutting and mounting in the diffusion cell. The tritiated water integrity testing methodology was also applied to these membranes which would, presumably, assist in the removal of water soluble enzymes present in the upper skin layers. If this is considered, with the absence of the relatively thick dermal layer, then

catabolism of permeant is not presumed to be a significant problem in the use of separated pig skin in the diffusion cell system. This postulation was verified by the absence of degradation products in the analysis of receptor chamber fluid.

Human Skin

From the outset it was apparent that obtaining samples of human tissue would pose the biggest problem if a full comparison of membranes was to be executed. While it would have been easy to simply omit this membrane from the comparative studies, its inclusion adds a certain degree of validity and relevance to the results obtained. Furthermore, very useful comparisons could then be made not only between the permeation results of human and laboratory animal tissue in this particular diffusion cell, but also between results from the *in vitro* diffusion experiments and *in vivo* bioassays using commercial products. Parallelism between the two experimental methods could then be assessed. The use of human tissue was, therefore, attractive from a number of viewpoints.

Human tissue is usually obtained from cadavers at autopsy, or from surgical amputations. This source was investigated and disfavoured because of the lengthy procedure involved in the evaluation and processing of applications by the authorities. The cantharidin blister technique reported by Kligman and Christophers *(157)* appeared to be a useful alternative source of stratum corneum plus epidermis from living volunteers. The method is very useful in that fairly large sheets of horny layer (several centimetres in diameter) may be prepared for diffusion experiments *(162)*. The methodology may be modified to produce tissue samples of the appropriate size to suit the diffusion cell in use. In addition, several samples may be obtained from the same donor, and from the same anatomical region of each donor so that reliable, comparative studies may be conducted. Furthermore, these intraepidermal blisters are obtained with a minimum of discomfort to the donor and, if the resultant wound is well cared for, no permanent scarring of the skin occurs on healing.

The cantharidin blister technique, therefore, appears to be extremely useful in the isolation of uniform sheets of human epidermal tissue. It is unfortunate that a chemical means of procurement had to be adopted for this tissue, however a number of reports appearing in the literature indicate that the blister technique does not significantly alter the permeability characteristics of the barrier layer *(157,174)*. Furthermore, the necessity for dermatome usage in sample preparation was avoided.

Donors were chosen from a panel of volunteers and thoroughly briefed on the methods to be employed in the acquisition of the skin samples and on the care to be exercised in treating the subsequent wound. All donors fully understood that they could withdraw their services at any time without prejudice. The inner aspect of the forearm was chosen as the site for sample removal as this is the region used by a number of corticosteroidal bioassays, including the blanching assay. By using skin from the same anatomical site, valid comparisons could be made between *in vitro* results and those of the bioassay as regional variations in skin permeability would not be a predominant discrepancy in the methodology.

A 0.2% solution of cantharidin (Sigma Chemicals, U.S.A.) was prepared in ethanol/water (60:40). A pure organic solvent, such as acetone used by Kligman and Christophers, was avoided as a vehicle for the cantharidin because of the possible deleterious effects it may have on the biochemical composition of the stratum corneum. Ethanolic solutions have been shown by several studies to have an insignificant effect on the barrier potential of the skin after prolonged contact *(24)* and this solvent was, therefore, chosen for the delivery of the cantharidin onto the skin. A plastic cylinder (2 cm in diameter by 1 cm tall) was used as a shroud to demarcate the skin area for application of the cantharidin solution, and to contain the spread of the solution to a specific area. Hirsute areas of the forearm were avoided wherever possible. Cantharidin solution (0.15 ml) was applied to the skin within the cylinder using a transfer pipette (Brand, West Germany) and spread evenly using the pipette tip. The fluid layer was then evaporated to dryness under a stream of warm air. A cylinder of larger area (approximately 4 cm in diameter) was then placed around the application site and taped in position, the smaller cylinder having been removed. The site remained in this condition for approximately six hours, the larger cylinder preventing contact of clothing with the applied film. After this period the volunteers reported a distinct itch sensation at the application site. The large cylinder was removed and the site covered with several layers of water-soaked cotton wool, extending the coverage beyond the application site. This cotton wool was then covered by overlapping layers or impervious tape (Blenderm, 3M, South Africa) which, together, would form a fully hydrated, occlusive barrier over the application site. Kligman and Christophers report that they occluded the sites with soaked cloth immediately after drying of the deposited cantharidin film. We found this method to be less satisfactory in the production of uniform blisters than when the site was left open to the atmosphere for several hours as previously described, possibly due to adsorption of the chemical onto the cotton wool thereby reducing its concentration on the skin.

The occlusive layers were left in position overnight and the blisters were presented for excision approximately 20-24 hours after initial application of the cantharidin solution. The arm of the volunteer was positioned horizontally, inner aspect uppermost, and turned slightly so that the blister surface was inclined away from the horizontal. This caused the serous fluid to drain and bulge the lower region of the blister (see Plate 8). A scalpel incision was then made into the upper, flattened extremity of the blister surface. Using surgical scissors, the membrane was liberated in both directions from this point of incision along the blister circumference, detaching as close as possible to the point of adherence of blister membrane to normal skin. The distention caused by the serous fluid prevented collapse of the blister surface onto the underlying epidermal/dermal tissue which greatly hinders excision of the blister membrane as lifting with forceps is then required prior to scission. In this manner the membrane could easily be excised and peeled from the forearm with a minimum of discomfort to the donor. Once totally removed, the membrane was spread on a dissection tile, epidermal side uppermost, and hydrated with purified water.

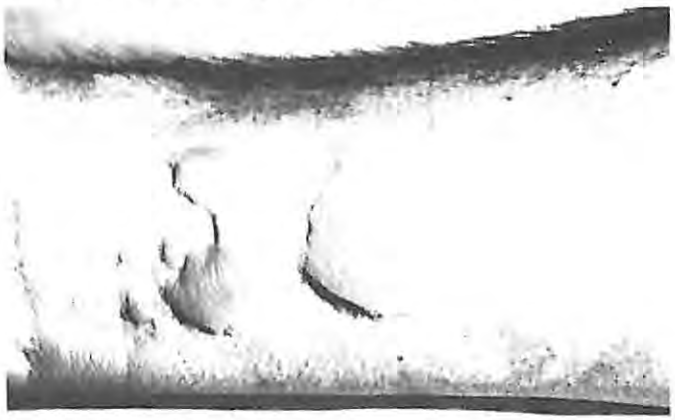


PLATE 8: Appearance of fluid-filled cantharidin-induced blister on donor forearm prior to excision.

The skin surrounding the excision site was thoroughly cleaned and the wound irrigated with purified water to remove any traces of cantharidin which may have been present in the serous fluid. The region was dried under a stream of cool air and either an antibacterial agent was applied (mercurochrome, Alpha Pharmaceuticals, South Africa or New Skin, Beige Pharmaceuticals, South Africa) or the area was left open to the atmosphere for several hours before a dressing was applied, according to the preference of the donors. It was generally concluded that the synthetic polymer dressing (New Skin), applied by aerosol application in a benzyl alcoholic solution, provided the best protection for the wound and was re-applied for several days after the excision. However, being alcohol based, this aerosol generated a transient stinging sensation, and slight lacrimation usually, on initial application,

but was requested by the majority of the volunteers. Furthermore, the alcohol base was useful in sterilizing the epidermal surface and, thereby, preventing infection of the wound. Each volunteer was given a supply of emollient cream to apply to the scab tissue once it had formed, to maintain the hydration and flexibility of the region during the healing process. The scab remained intact for approximately 7-10 days after which the new epidermal proliferation was visible.

Theoretically, treatment and care in this fashion should leave little scar tissue once the wound has healed, primarily because the blister is induced intraepidermally and does not disturb the basement membrane. However, donor care for the wound was not ideal in many instances—despite repeated counselling. Scab tissue was allowed to desiccate and crack with subsequent serous weeping in some cases, while in others subsequent physical trauma dislodged or disrupted the scab surface. In these cases a darkening of the healed skin was experienced, corresponding to the blister size and shape. This hyperpigmentation gradually faded and had totally disappeared seven to 12 months after blister excision. The hyperpigmentation was more pronounced in some volunteers than in others and, interestingly, the membranes from these volunteers also demonstrated greater permeability characteristics to tritiated water, but not corticosteroid, passage.

The excised blister surface, spread on the dissection tile, tends to curl under the influence of the epidermal tissue clinging to the undersurface of the stratum corneum. Irrigation with purified water and soaking for approximately 30 minutes allows this loose, adherent layer to be teased away from the stratum corneum using two pairs of forceps, one pair to secure the horny layer and the other to manipulate the epidermal debris. This cleaning step can be undertaken with a surprising degree of vigour without inducing any damage to horny layer, even though the latter appears extremely fragile. Once this underlying tissue has been removed, the stratum corneum sheet is flat, translucent and generally occupies an area slightly larger than that of the excised blister. The undulating rete ridges of the epidermis tend to wrinkle the upper stratum and the removal of the former allows the horny layer to spread flat, the smoothing of the wrinkles generating a slightly greater surface area. The stratum corneum may then be trimmed and mounted directly in the diffusion cell or air dried and stored in the desiccated state, without refrigeration, until required. Membranes dried in this manner were soaked in purified water for at least six hours, prior to mounting in the diffusion cell, for rehydration and to prevent membrane distortion due to tissue swelling if dry-mounted in the cell. As with the other biological membranes

reported above, exposure to the aqueous receptor phase during the tritiated water validation may extract enzymes present in the stratum corneum *(48)*. The relatively thin membrane and lack of epidermal tissue are, therefore, not assumed to present a significant metabolic potential to the subsequent corticosteroid permeants. Plate 9 shows the superficial and sectional appearance of the membrane prepared in this manner.

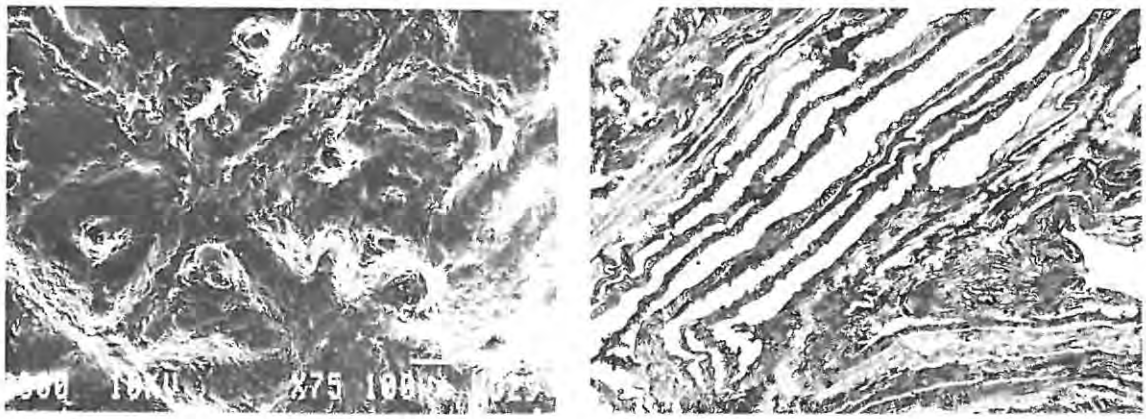


PLATE 9: Topography and transverse section of human stratum corneum obtained from a cantharidin-induced blister.

This method of human skin acquisition was chosen in preference to sampling from cadavers or amputated limbs because of the lengthy paperwork involved in the latter. In retrospect, the cantharidin blister method appears to be as intensive in planning and in co-ordination of the prompt, repeated appearances by the volunteers. Furthermore, the occasional necessity to re-apply cantharidin solution to small, adherent areas of partially formed blisters, delayed the initiation of several diffusion experiments. Refinement of the above methodology alleviated some problems to a certain extent. One modification which was promulgated in certain cases was the application of cantharidin solution to larger areas (approximately 3.5 cm in diameter) which produced sufficient membrane for three diffusion cells. In this manner the reporting of the volunteer to the laboratory did not have to be as frequent, a factor which was well received by the donors. These problems would not have presented themselves had a supply of excised skin, from cadavers or amputations, been available, in frozen storage, for immediate use by the researcher. For these reasons, it is suggested that samples from cadavers be acquired in future in preference to the adoption of the cantharidin blister technique.

Egg-Shell Membrane

The shells of freshly drained eggs were soaked in purified water overnight after which the membrane could easily be lifted from the inner surface of the shell. The membranes were immediately mounted in the diffusion cells and no

method of frozen storage was employed. The topographical and transverse sectional nature of this membrane is illustrated in Plate 10. The dissimilarity between this medium and animal skin is apparent if these photographs are compared to Plates 6-8.

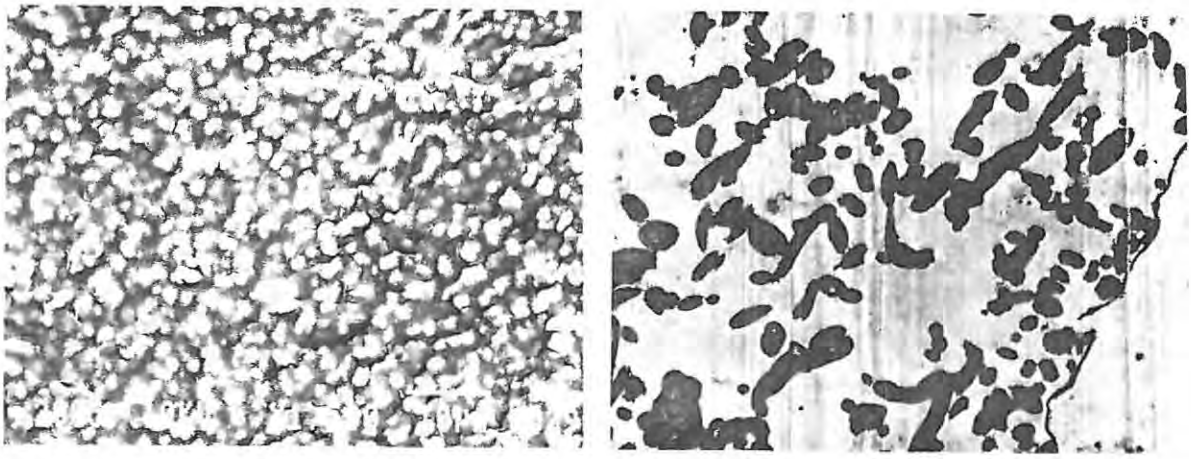


PLATE 10: Topography and transverse section of egg-shell membrane.

3.4 MEMBRANE INTEGRITY VALIDATION

From the foregoing descriptions of membrane characteristics it is obvious that one important facet of the in vitro diffusion process is the presentation of a uniform, integral medium for permeation by the corticosteroid. Any flaw in the membrane barrier will manifest itself as a facile portal for the passage of the diffusant and abnormally high flux rates will result. The methods of isolation and preparation of the biological membranes are intensive, subjecting the membrane to both physical and chemical trauma in many instances. Damage caused to the skin tissues during stratal separation or cleaning is generally covert and may not be detected even under microscopic examination. A simple loosening of the stratum corneum layers, without actual separation, may increase the permeability of the membrane several fold, without any obvious, visual, porous damage. Diseased skin, even though undamaged by the preparative techniques, may exhibit abnormally high permeability characteristics and these samples must also be screened for and excluded from diffusion experiments. Similarly, cleaning or inadvertent dehydration of synthetic media may result in polymer fracture with a concomitant decrease in barrier potential. Thus, some reliable form of assessment of the integrity of the prepared membrane is vital, the omission of such a validation procedure may shadow diffusion results with doubts concerning the diffusive integrity of the media used.

BACKGROUND

Light microscopy has been used throughout this experimentation to examine the surface topography of the synthetic membranes prior to mounting of the medium in the diffusion cell. Gross damage to the membrane continuity or hypernormal pore sizes were searched for but none were located in the uniform commercial products. As previously suggested, damage to biological tissue is generally more subtle and usually concealed from light microscopic disclosure. Therefore, a more sensitive method had to be employed for validating excised skin samples. The best manner to check the diffusive characteristics of a membrane is, obviously, to carry out a diffusion experiment. If "normal" flux rates are known for a particular diffusant passing through a specific membrane then the observation of hypernormal flux values during a validation run may suggest the medium is damaged and not representative of the membrane population mean. For such a preliminary validation, a diffusant must be chosen which is as innocuous as possible, it should interact minimally with the tissue and generate no irreversible changes to the barrier. Similarly, its presence in the membrane should not influence the passage of subsequent diffusants investigated.

Water is a diffusant which appears to satisfy these criteria perfectly. It is a normal constituent of the skin and, therefore, does not represent a foreign chemical to which the tissue is exposed. Water applied to the skin rapidly penetrates the superficial layers and equilibrates with bound and solubilized water within the cell strata. No specific saturation of binding sites is necessary in the stratum corneum or epidermis which results in rapid emergence of the diffusant molecules from the distal surface of the membrane. Diffusant water molecules that remain in the tissue are in equilibrium with endogenous water and, thus, the barrier potential of the skin is not affected in any manner by the passage of the validating diffusant. Additionally, the permeation behaviour of subsequently applied chemicals should not be altered as a result of the previously conducted water diffusion experiment. Several studies have demonstrated that the diffusion of water *in vitro* closely parallels that *in vivo (17,112,154)* and the water permeability coefficient has been used as an index of barrier function by a number of researchers *(15,112, 152)*. Hence, the use of water as a validating species to assess membrane integrity prior to the initiation of corticosteroid diffusion experiments appears to be ideal from a number of viewpoints.

The distinction between applied and endogenous water may pose a problem in the receptor chamber analysis. Radiolabelling of the diffusant appears to be the most facile solution to this problem and is convenient, additionally,

in that assay of the receptor phase is relatively simple using radiocounting techniques. Hydrogen-3 (tritium) labelled water is available commercially and has been adopted for these diffusion experiments by a number of researchers *(18,19,140,159,160,267)*. The substance is handled with relative ease and has the additional advantage in producing β-emission of relatively low energy (0.0186 MeV at 100% isotopic abundance). The dangers in radioactive exposure of operators to this moiety are, therefore, less than if more energetic radionuclides are employed. In using this species the validation procedure would, in essence, measure the rate of permeation of tritiated water from a solution of this permeant in unlabelled water, to a receptor fluid of unlabelled water, through the membrane under investigation. This measured permeation rate could then be compared to published values for the particular membrane to assess its deviation, if any, from normal barrier potential. Substantial deviation would suggest the occurrence of damage during the preparation procedures or abnormal skin sources (*eg.* diseased skin).

METHODOLOGY

A 1-ml aliquot of tritiated water, rated at 5 mCi ml^{-1} (185 MBq ml^{-1}) activity (code TRS.3, Amersham, U.K.) was diluted to 1000 ml with purified water (high-performance liquid chromatographic grade, prepared as in section 3.1) producing a final donor solution of tritiated water in purified water vehicle of activity 5 μCi ml^{-1}. The tritium radionuclide has a half-life of 12.43 years indicating that solutions would decay insignificantly, even during several weeks of experimentation. A scintillation fluid (cocktail) is required which will convert the radioactive decay into light emission, subsequently measured by the liquid scintillation counter. Small volumes of receptor fluid would be withdrawn at each sampling interval requiring that this conversion be extremely sensitive to trace amounts of radionuclide. Pseudocumene is a fluor solvent which will carry out this conversion with high efficiency and is favoured in modern scintillation cocktails. A commercial cocktail was chosen containing this solvent (Ready-Solv HP, Beckman, U.S.A.) on the basis that the receptor chamber sample would be aqueous, of small volume, and cocktail volume should be as frugal as possible. This scintillation solution will usually hold up to 15% aqueous sample load in a clear, continuous emulsion without two-phase breaks. Counting efficiency was investigated using the donor tritiated water solution (5 μCi ml^{-1}) and varying volumes of this scintillation cocktail. It was found that the greatest efficiency was obtained when 40 μl of tritiated water solution was added to 5 ml of counting solvent, smaller volumes of the latter resulted in poorer counting results. The literature

would indicate that, at this sample load, the tritium counting efficiency would be approximately 48% but a calculation of the absolute value of disintegrations per minute is unnecessary as a simple ratio of the measured counts per minute (CPM) is used in the computations, as detailed later.

A sample of animal or human skin was mounted in the diffusion cell as described in section 3.5, ensuring adequate sealing of the flange surfaces to prevent fluid leakage. An accurately measured volume of purified water (approximately 10 ml) was pipetted into the receptor chamber of the cell, the magnetic stirrer bar inserted into the chamber and the cell lowered into the water bath for temperature equilibration at 35 °C. After approximately 30 minutes, air bubbles that had formed in the receptor chamber during the heating of the water were expelled via the sampling port by carefully tipping the cell. A 40-µl sample of receptor chamber water (blank) was then withdrawn (fixed volume transfer pipette, Brand, West Germany) and the volume replaced with fresh, purified water. The blank aliquot was transferred directly to a polyethylene minivial (Mini Poly-Q, Beckman, U.S.A.) containing 5 ml of scintillation cocktail. A volume of tritiated water solution was then pipetted into the donor chamber of the diffusion cell and the time noted. The exact volume of this aliquot is not important as the donor solution presents an infinite dose of permeant to the membrane surface which is insignificantly depleted during the period of monitored diffusion. The cell was lowered into the thermostated water bath and the stirrer set in motion. After three minutes had elapsed for equilibration, a 40-µl aliquot of tritiated water solution was similarly withdrawn from the donor chamber and transferred to a minivial containing scintillation cocktail. This would represent the initial donor concentration of permeant.

Typically, 40-µl portions of the receptor phase were withdrawn at 30-minute or hourly intervals for six hours, with purified water replacement, and transferred to scintillation vials for counting. The vials were capped and vortexed for 30 seconds to ensure homogeneous dispersion of the emulsion. Radioactivity was assessed in a Beckman LS2800 liquid scintillation counter immediately after withdrawal of each sample aliquot from the diffusion cell. The instrument was set to count each sample for a three minute period, and the counting was repeated 10 times for each vial after which the average counts per minute was calculated. A tritium sample was withdrawn from the donor compartment at the end of the diffusion experiment and its count value compared to that of the donor sample withdrawn at the start of permeation. This comparison would validate that insignificant depletion of the donor solution had occurred during the course of the diffusion, maintaining steady-

state conditions. Anomolous discrepancies between the loss of permeant from the donor chamber and appearance in the receptor solution could also be investigated. In all cases these donor values were insignificantly different to one another and each averaged approximately 2.5 x 10^5 CPM. Counting of the blank receptor sample, taken before application of the donor solution, would assess the operation of the instrument and, typically, measured the activity of the blank sample emulsion to be approximately 15 CPM.

RESULTS

For each membrane a graph was plotted of the quantity of permeated tritiated water (as represented by the counts per minute in the receptor fluid) *versus* time and the rate of absorption was calculated from the linear portion of the graph. A permeability coefficient could then be calculated by the quotient of the absorption rate and applied concentration according to Equation 15 *(137,184)*:

$$P = (CPM_2 - CPM_1) / (t_2 - t_1) \times V / (A \times CPM_d) \quad \text{Equation 15}$$

where P is the permeability coefficient, CPM_1 and t_1 are the counts per minute value and time, respectively, at the start of steady-state diffusion, CPM_2 and t_2 are the count per minute value and time at the end of the diffusion experiment, V is the volume of the receptor chamber solution, A is the diffusion area of the membrane and CPM_d is the count per minute value of the donor tritiated water solution.

Permeability coefficient values reported in the literature for normal (undamaged) human skin vary between 0.9-2.5 x 10^{-3} cm h^{-1} *(15,78,112, 152,183,184)*, although most of these values are for permeation through excised abdominal skin. Dugard and coworkers *(19,160)* report that they rejected all membranes as damaged having a permeability coefficient of 1.5 x 10^{-3} cm h^{-1} or greater. As with most studies involving biological tissue, an extremely wide variability in permeation is expected: Bronaugh *et al. (152)* observed a five fold variance in water permeation in their studies, 33% of their prepared skin samples exhibited a permeability coefficient greater than 2.5 x 10^{-3} cm h^{-1} and were rejected on that basis. The results of this research demonstrate that permeability coefficients vary substantially, within the same order of magnitude, between donors, but are fairly constant for multiple skin samples taken from the same donor. In some cases the measured permeability coefficients have been greater than the accepted cut-off value for rejection. It

does not seem probable that every membrane removed from each of these donors would be damaged, to almost the same extent, more likely this is simply an expression of interperson variance and it is, therefore, proposed that absolute rejection of these samples is unnecessary. A measured permeability coefficient of 6×10^{-3} cm h^{-1}, for example, does not necessarily indicate that the skin sample is damaged, especially if this value is not markedly variant from the mean value for samples taken from that particular donor. Thus, data from a number of excised samples is required so that the mean permeability coefficient for each subject may be established. Only once this database has been formulated, may samples be rejected on the basis of high coefficient values. Table VII lists the average permeability coefficients (P) obtained by tritiated water validation in this manner for forearm skin samples from five human volunteers used in this research. The samples were excised and prepared as reported in section 3.3.

TABLE VII: Average permeability coefficients for tritiated water diffusing through human skin at 35 °C.

Donor	Sex	Age (y)	P x 10^3 (cm h^{-1})	SD x 10^3 (cm h^{-1})	n
1	M	24	1.9985	0.8982	12
2	M	23	4.9413	1.2806	12
3	F	23	3.6133	0.4208	4
4	M	35	2.5543	1.4058	3
5	M	38	1.5461	0.3973	8

These values are in fairly close agreement with those reported in the literature but are higher, in some cases, than values classically taken to designate barrier integrity. They are, for example, slightly higher than the 0.84×10^{-3} cm h^{-1} value reported by Scott *et al. (160)* for separated human epidermal membrane although these authors do not report how many different donor cadavers were used for their determinations. Notwithstanding their magnitude, on the basis of the above proposition, these values were taken to be representative of undamaged membrane sampled from a number of donors exhibiting normal interperson variation. Permeability coefficient values approaching an order of magnitude greater than the norm were regarded as sufficiently deviant to be excluded from the experimentation. None of the prepared membranes tested approached this degree of permeability.

After radioactive counting of the first receptor-phase sample, it was immediately apparent whether or not the membrane was damaged, the half-hour sample for undamaged human skin would typically record a count value of approximately 80-150 CPM. Damaged samples, on the other hand, would record values approximately three orders of magnitude higher, exemplifying the more facile permeant passage. Values obtained for the animal tissues were of the same magnitude. It was, therefore, possible to detect and reject a damaged

skin sample after the initial 30 minutes of tritiated water diffusion. This is obviously beneficial as time is not wasted with lengthy validation procedures. Skin samples that demonstrated low initial radioactive permeation (suggesting they were undamaged), were tested for at least six hours for a valid estimation of the permeability coefficient. Table VIII lists the average permeability coefficients for hairless mouse and pig skin, prepared as detailed in section 3.3, and, for comparison, the average values of the human skin samples.

TABLE VIII: Average tritiated water permeability coefficients diffusing through human and animal skin at 35 °C.

Donor	Sex	Age (weeks)	Skin Source	P x 10^3 (cm h^{-1})	SD x 10^3 (cm h^{-1})	n
Hairless	M	9-10	Abdominal	1.7111*	1.4983	18
Mouse	M	9-10	Dorsal	1.5986*	1.1221	21
Pig	M	0-1	Abdominal	8.4813	12.2060	39
Human	M	Adult	Forearm	2.9159+	1.7744	35
	F	Adult	Forearm	3.6133+	0.4208	4

* + values not significantly different.

The average permeability values reported here are slightly lower for the hairless mouse and slightly higher for the pig skin than those reported by Scott *et al. (184)* (Table I), however all are of the same order of magnitude. Full thickness pig skin was used by Scott and coworkers which may account for their lower permeability value (1.8×10^{-3} cm h^{-1}). Furthermore, the nonideal method of pig stratum corneum preparation in this study, described in section 3.3, may account for the large permeability coefficient standard deviation for these samples. The presence of the relatively large hair follicles and shafts makes the heat separation of the pig stratum corneum impractical. Traumatic rupture of these structures resulted in the rejection of approximately 10% of the prepared membranes, impairment being indicated by high tritiated water permeability. Scott *et al. (160)* have used 2.0×10^{-3} cm h^{-1} as the datum permeability value for rejection of tested rat skin. The hairless mouse skin verified in this research had permeability values falling within this range and were therefore considered undamaged. Moreover, these results are in agreement with the findings of Behl *et al. (29,190)* in that the permeability of dorsal and abdominal hairless mouse skin is insignificantly different.

Following the preliminary tritiated water validation procedure, the main diffusion experiment using betamethasone 17-valerate was conducted as described in section 3.5. Initially, human stratum corneum membranes were re-evaluated by identical tritiated water experimentation at the end of the corticosteroid diffusion run. The measured radioactivity in these cases was generally two orders of magnitude, or more, greater than the preliminary

values observed for the same membrane. This indicated that some permanent modification of the permeability barrier had occurred during the relatively long (three or more days) diffusion experiment. Furthermore, these values indicated that re-use of the same skin sample for subsequent diffusion runs was not feasible.

CORRELATION WITH OTHER DIFFUSANTS

Langguth *et al. (103)* and other workers *(268)* have suggested that a feasible approach to reducing the large number of skin samples normally required in testing transdermal drug permeation, because of their intersample variability, may be to calibrate the diffusive characteristics of the tissue with respect to a standard diffusant molecule. A database of permeability values is initially established by measuring the flux of the standard compound through several membrane samples. A minimum, maximum and mean flux value may then be specified for the standard and this information is stored as a prospective reference. Subsequently the permeation of the test drug and the standard is measured through each of relatively few membrane samples. If it is established that a good correlation exists between the permeability of the standard and that of the investigative drug then the minimum, maximum and mean permeabilities of the test drug may be estimated by interpolation of the reference standard data range. In this manner only a few membrane samples are required to reliably characterize the diffusive nature of the test drug.

If a correlation of this nature could be demonstrated or the permeability of tritiated water and betamethasone 17-valerate then this preliminary validation step would prove even more useful. Not only would it demonstrate the integrity of the membrane and serve as a useful leaching step for the removal of metabolic enzymes from the tissue but, additionally, it may provide an estimation of the expected permeation of the corticosteroid through each sample. If, however, different pathways do exist for the permeation of chemicals of differing polarity, then high water permeability may not necessarily reflect high permeability to more lipophilic moieties. Bronaugh et al. *(152)* have conducted a similar investigation in which the permeation of tritiated water and a number of compounds of diverse nature was measured through human skin samples. They observed that in all cases membranes more permeable to water were also more permeable to the test compound, regardless of the polarity of the latter. Comparing experimental values to those from control membranes of low water permeability, they found the magnitude of the increase in water permeation was significantly different to the increase in test compound permeation only for cortisone and DDT of the seven permeants

studied. The relative increase in cortisone permeation was less than the increase in water permeation, with respect to the control membrane values, and that of DDT was greater. Bearing this result for cortisone in mind, it is interesting to note that the increase in water permeability was not significantly different from the increase in testosterone permeability. This tends to suggest that certain of the corticosteroids may exhibit unique permeation characteristics. The authors conclude that the monitoring of water absorption appears to be a good indicator of general changes in barrier integrity for compounds of diverse water solubility.

To investigate possible correlation in this research, the permeability coefficients recorded for tritiated water and, subsequently, betamethasone 17-valerate diffusion through human and animal skin samples were compared and the results are depicted in Figure 22. The points indicate the permeation data from stratum corneum samples of three human donors and four porcine samples. The experiments were carried out at 35 °C, the tritiated water permeation was monitored as described above and the steady-state diffusion of the 0.1% corticosteroid solution in isopropyl myristate was monitored as described in section 3.5. As is evident from the scatter of the points, there is no apparent correlation between the permeability coefficients of the two diffusing species. While it must be borne in mind that only relatively few samples of skin from a small donor population have been tested, it is assumed that if correlation does exist this would have manifested itself more clearly in the results.

The corresponding results for hairless mouse skin are an order of magnitude greater for the corticosteroid permeability coefficient, even though the tritiated water values are fairly similar to human or pig skin. Table IX lists coefficients for tritiated water and betamethasone 17-valerate permeating through samples of full thickness mouse skin from different anatomical regions of a single animal.

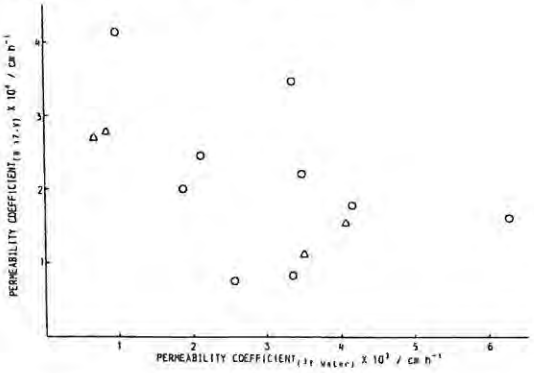


FIGURE 22: Comparison of betamethasone 17-valerate and tritiated water permeability coefficients for human (○) and weanling pig (△) stratum corneum samples.

TABLE IX:
Permeability coefficients for tritiated water and betamethasone 17-valerate diffusing through male hairless mouse skin at 35 °C.

Skin Source	P(B17V) x 10^3 (cm h^{-1})	P(T Water) x 10^3 (cm h^{-1})
Abdominal	2.2598	3.5980
Abdominal	2.5062	0.7467
Dorsal	2.3830	2.1742

These results are also interesting in that corticosteroid permeability is fairly constant for all skin samples, while tritiated water permeability values measured through the same samples are obviously variant. This further suggests that little correlation exists between the permeabilities of these two moieties. The vastly differing hydrophilic characters of the permeants may account for poor correlation in permeability. Langguth *et al. (103)* used glyceryl trinitrate as the standard diffusant and found that the permeabilities of benzoic acid, ephedrine, fursemide and caffeine correlated closely while the quaternary compound trospium chloride demonstrated extremely poor agreement with the flux of the standard. Differing diffusion pathways for the hydrophilic and lipophilic moieties may also play a significant factor in this discrepancy, although it has been suggested that the hair follicles and shunt routes of permeation play an insignificant part in the percutaneous absorption of water *(184,269)*. From these results it would appear that the diffusive nature of water molecules is too dissimilar from that of larger corticosteroid diffusant for flux values of the two species to parallel one another.

CONCLUSIONS

In summary, the monitoring of tritiated water permeation through the prepared biological membranes is a very useful, rapid method of validating the integrity of the barrier medium and the use of this methodology ensures that damaged tissue is excluded from drug diffusion experiments. However, the permeability coefficients obtained for the tritiated water permeation give, apparently, very little indication of the subsequent permeability of betamethasone 17-valerate through the same membranes. There is no obvious correlation between the permeability coefficients of water and this corticosteroid for the membranes tested. A further factor worthy of consideration is that this radionuclide validation procedure exposes the membrane to an aqueous environment for a protracted period. This generates full, nonphysiological hydration of the tissue which may enhance its permeability, not only to the tritiated water species, but also to any subsequently applied diffusants. However, this situation parallels percutaneous absorption experiments, both *in vitro* and *in vivo*, that are carried out under conditions of rigorous occlusion, a situation in which similar enhancement of permeability would be expected *(270)*. One possible method of avoiding this full hydration of the skin is to follow the methodology proposed by Bronaugh and coworkers *(152)*. They allowed an aliquot of donor tritiated water to contact the surface of the membrane for only 20 minutes, after which time excess water was blotted away. Results from this finite-dose type experiment (measuring percentage of the applied dose

absorbed) were shown to correlate extremely closely with long contact time, steady-state diffusion validations as described above. The integrity validating ability of the two methods is, therefore, equivalent but the short exposure time of the proposed method would not generate full hydration of the tissue.

3.5 PROTOCOL FOR TYPICAL *IN VITRO* DIFFUSION EXPERIMENTS

STEADY-STATE DIFFUSION EXPERIMENTS

The monitoring of steady-state permeation of corticosteroid from solution to solvent requires the horizontal mounting of two diffusion chambers, in mirror image, separated by the membrane under investigation. This membrane would typically be held firmly in place between the ground glass flange surfaces of both diffusion chambers, secured in position with layers of Parafilm to create a water-tight seal, and the chambers seated firmly between the jaws of a common laboratory apparatus clamp (see Figure 8). A number of these clamps, and their diffusion cells, could be positioned over a single laboratory stirrer, for replicate experiments, so that the diffusion chambers and membranes were totally immersed in the water bath. The membranes used for experimentation were prepared as described in section 3.3 prior to mounting in the cell system and the skin media were initially subjected to the tritiated water validation procedure (Section 3.4) after which each chamber was emptied of water and the interior dried under a stream of nitrogen. The skin media were mounted with their outer stratum corneum surfaces orientated toward the donor chamber so that drug passage would follow the same direction as that experienced *in vivo*. The separated stratum corneum membranes were more easily manipulated if they were spread on grease-proof paper or polythene sheeting, this would prevent the media from curling and greatly facilitate handling and cutting.

Prior to the commencement of the steady-state diffusion experiment, a specific volume of purified isopropyl myristate was pipetted into each chamber of the assembled system, 10 mm teflon-coated magnetic stirring bars were inserted into each chamber and the sampling tubes stoppered. The bichamber cell was then immersed in the thermostated water bath and the stirrers set in motion to allow equilibration of the membrane with the bathing isopropyl myristate. After approximately six hours equilibration, the fluid was

completely removed from the donor chamber and replaced with a specific volume of corticosteroid donor solution. A 0.1% solution of betamethasone 17-valerate was prepared in purified isopropyl myristate, with the aid of ultrasonification, and this was used as the donor vehicle for the drug. This procedure initiated the diffusion experiment and was recorded as time zero. After approximately two minutes of immersed stirring, 40 µl-aliquot samples were withdrawn from both the donor and receptor chambers using a fixed volume transfer pipette (Brand, West Germany) and the fluid volumes replaced with 0.1% drug solution and purified isopropyl myristate, respectively. In all cases, sampling and replacement of the chamber fluid was effected by inserting the tube of the pipette through the hole in the teflon sampling port stopper, after removal of the outer sealing disc. These primary aliquots represented the initial donor and receptor chamber fluid concentrations of betamethasone 17-valerate. Thereafter, (at regular, predetermined, intervals) further 40 µl-aliquots were sampled from the receptor chamber solution, with volume replacement. The intervals between sampling should be relatively short at the start of the experiment, so that the nonlinear lag time phase may be fully characterized, and this interval may be extended once steady-state permeation is achieved. All these samples were stored under refrigeration in narrow bore glass tubes until assayed by high-performance liquid chromatography (Section 3.2). At the end of the diffusion experiment a further aliquot was taken from the donor chamber solution, which represented the final donor concentration.

The vertical mounting of the membrane prevented air bubbles from lodging against this medium and, therefore, no further manipulation of the cell was required during the course of the experiment. The water level in the immersion bath, the water temperature and the revolution rate of the stirrers were checked at regular intervals to ensure that external variables were not introduced into the experimentation.

In this fashion the donor corticosteroid solution would deliver permeant across the membrane to the equally agitated solvent over the time-course of the experiment, both chambers being immersed in a water bath at controlled temperature. In these tests of solution to solvent diffusion, the intrinsic diffusivity of the drug through the membrane, both during the lag phase and at steady-state, could be fully elucidated as there are no topical formulation constituents present that may alter the barrier properties of the membrane or enhance penetration.

IN VIVO-MIMIC DIFFUSION EXPERIMENTS

The experimental procedures for this investigation are essentially the same as those described above, with the exception of the cell orientation. A single, vertically mounted diffusion chamber may be used to monitor the permeation of corticosteroid from a semisolid, topical formulation, through the membrane under investigation, to the isopropyl myristate receptor phase. In these instances the donor formulation is contained within a small, cylindrical donor shield (Figure 9), which replaces the donor chamber of the steady-state experiments. This donor shroud has a lower ground glass flange surface which secures the membrane to the flange surface of the receptor chamber, the two portions of the cell, again, being sealed by Parafilm and fixed together by apparatus clamp. For this permeation chamber configuration, the procedures of membrane preparation, mounting, integrity validation and equilibration with isopropyl myristate solvent were identical to those described previously for the steady-state experiments.

The *in vivo*-mimic diffusion experiments are initiated by the application of an infinite dose of topical formulation to the donor side of the membrane, spreading the donor vehicle evenly with a glass rod and ensuring intimate contact of the entire diffusion area with the donor formulation. While easily accomplished with the solution, lotions and creams, gentle heating of the viscous ointment dosage forms facilitated this spreading procedure. The donor cylinder, containing the formulation, was then covered by a layer of Parafilm to prevent evaporation of constituents from the applied vehicle. The receptor chamber, containing the stirrer bar, was lowered into the water bath to the level of the membrane and positioned, using the apparatus clamp, over the magnetic stirrer. In this manner the donor formulation itself is not heated by the water bath. A temperature gradient is, thus, allowed to establish itself between the receptor surface of the membrane and the ambient atmosphere, as would exist under conditions of clinical usage of the formulation.

After approximately two minutes of immersed stirring an initial 40 µl-aliquot was removed from the receptor chamber and the volume replaced with purified isopropyl myristate. Thereafter, as before, at predetermined intervals, further aliquots were removed from the receptor chamber, with volume replacement, and the samples stored under refrigeration until assayed. As the membrane is mounted horizontally in this study, any air bubbles that form will accumulate against the undersurface of the medium. These bubbles will require expulsion as they form if the area available for permeation is not to be reduced significantly, with a concomitant reduction in the drug flux rate. Regular inspection of the membrane undersurface is therefore required

throughout the course of the experiment and tipping of the diffusion cell, to expel the bubbles through the sampling side-arm, undertaken when necessary. This is a fairly facile procedure which is quickly accomplished and does not upset the permeation or temperature equilibrium.

In this manner the full permeation profile of the drug from the topical formulation over a number of days may be gleaned. In most cases the magnitude of the permeation from these semisolid products is expected to be significantly less than that observed in the steady-state diffusion experiments using the solution donor vehicle. However, in the presence of potent penetration enhancers or agents that may denature the skin media, the permeation of corticosteroid from the topical formulations may exceed that from the simple isopropyl myristate solution. Examination of the permeation results indicate that this is achieved in certain instances.

SPECIFIC INVESTIGATIONS UNDERTAKEN

In both the steady-state and *in vivo*-mimic investigations, each variable was investigated by at least triplicate diffusion experiments as described above. Diffusion runs were repeated where results demonstrated abnormal variance so that final permeation rates could be quoted with a fair degree of confidence. After the initial cell design validation procedures, utilizing agitation and temperature variables as described in Section 2.3, a number of experiments were carried out to fully characterize the permeation of betamethasone 17-valerate from different donor vehicles (solution to ointment dosage forms) and through different membrane media (synthetic membranes to human stratum corneum).

Initially an investigation was carried out to determine the intrinsic diffusivity of the corticosteroid through various synthetic and biological media. Steady-state diffusion experiments were conducted, as described above, using a 0.1% solution of the drug in isopropyl myristate as the donor vehicle, purified isopropyl myristate as the receptor phase and the following barrier membranes: Nuclepore filter membrane, egg-shell membrane, cellulose dialysis media (soaked at various temperatures and boiled), Silastic silicone sheeting, full thickness hairless mouse skin, and weanling and human stratum corneum. The aim of this study was to possibly elucidate a synthetic or animal membrane exhibiting similar permeability characteristics to human tissue which may be used in place of the latter in laboratory diffusion experiments.

Thereafter, investigations were carried out to determine the permeation characteristics of the corticosteroid from various topical dosage forms (scalp application, lotions, creams and ointments) and the results using formulations

from different manufacturers were compared. Both synthetic and biological media were, again, used in this *in vivo*-mimic methodology, as described above. It was assumed that a good *in vitro* model for percutaneous absorption should be able to sensitively distinguish between the donor formulations of different manufacturers. This distinction has clearly been demonstrated *in vivo* by the human blanching assay. For example, results from the blanching assay may show three cream formulations from different manufacturers, containing the same corticosteroid in the same concentration, to exhibit three significantly different blanching profiles under identical investigation. By inference, this postulates that the bioavailabilities of the drug from the three products are significantly different, even though they are contained within similar cream formulations. A reliable *in vitro* diffusion cell system should equally be able to demonstrate these subtle differences in formulation release rates, or else its usefulness in diffusion experimentation is limited. This investigation would, therefore, be a further validation of the diffusion cell performance and would, ultimately, strive for correlation between *in vitro* data and *in vivo* blanching trial results.

The products tested in this study are detailed in Appendix 1, the drug permeation from three 0.1% betamethasone (as the 17-valerate) cream and ointment formulations (Betnovate, Glaxo, South Africa; Celestoderm-V, Scherag, South Africa and Persivate, Lennon, South Africa), two lotion formulations (Betnovate and Celestoderm-V) and one ethanolic scalp application (Betnovate) were investigated using silicone sheeting, full thickness hairless mouse skin, weanling and human stratum corneum as the barrier media. These commercial products represented the full spectrum of betamethasone 17-valerate containing topical formulations on the South African market. Finally, as a preliminary investigation, the permeation of hydrocortisone from two 0.5% cream formulations (Cutaderm, Scherag, South Africa and an experimental formulation, Lennon, South Africa) was also investigated using this *in vitro* diffusion cell system, and the results compared to blanching data. The permeation results from all these studies, and discussion thereof, are presented in Section 4.

3.6 THE HUMAN BLANCHING ASSAY

The human skin blanching assay has become the assessment method of choice for the rapid evaluation of the potency, bioavailability and absorption of topical corticosteroids. It has been developed in response to the accelerating use of these compounds over the last two decades which has necessitated

an accurate method for estimating their clinical efficacy and bioequivalence. The assay makes use of the vasoconstrictive side effect of the diffusing corticosteroids, once they reach the dermal vasculature, which induces a pallor or blanching of the skin at the site of drug application. The degree of blanching the skin exhibits correlates directly with the mass of drug which has penetrated to the vasculature. This phenomenon was first used by McKenzie and Stoughton in 1962 *(271)* to estimate the relative quantities of topically applied steroids absorbed by the skin. Since that preliminary investigation a number of experimental methods have been developed, evaluated and refined by several research groups in an attempt to improve the assessment technique *(211,272)*. The resulting methodology has been optimized to provide the most precise and reliable means of measuring corticosteroid potency and release from topical formulations *(273)*.

METHODOLOGY

The basic methodology for conducting the blanching trial using human volunteers encompasses the application of a known amount of drug onto discrete skin sites, allowing the corticosteroid vehicle to remain in contact with the skin for a specific period, removal of excess formulation and subsequent visual assessment of the degree of induced blanching over a prolonged time to yield a response profile.

The volunteers for the trial, usually 12 male and female Caucasians, are selected from a panel of subjects who are known to elicit a blanching response to a standard betamethasone 17-valerate cream. Volunteers taking systemic medication or who have used topical corticosteroids in the six weeks prior to the date of the trial are not included in the assay. All volunteers present themselves for assessment on the same day to minimize the possible environmental variables such as temperature and humidity which have been shown to influence the resultant blanching response.

With each volunteer, six adhesive labels, from which two independent 7 x 7 mm squares have been punched, are applied to the flexor aspects of each forearm, producing 24 discrete application sites per volunteer. The semisolid topical corticosteroid formulations to be applied to these demarcated sites are loaded into small bore, plastic syringes immediately prior to the trial and are extruded onto the skin via 25-gauge needles which have been cut to 5 mm length to facilitate extrusion. Usually four 7 mm stripes of formulation are extruded onto each site which delivers approximately 3.2 mg of product in a fairly reproducible fashion *(272)*. Lotion or solution dosage forms are applied via transfer pipette (variable volume, Brand, West Germany), a 5-µl

aliquot of liquid is usually applied, approximating 4 mg of formulation. The extruded formulations are evenly spread over the demarcated sites using a different glass rod for each preparation, or the liquid dosage forms are spread using the tip of the pipette.

Four different application patterns are used in any one trial to prevent the emergence of a recognizable pattern in the observed blanching response. The patterns are randomly assigned to the volunteers ensuring that the same pattern is not used on both arms of the same person. The patterns are composed in such a manner that each preparation to be evaluated is applied, an equal number of times, to sites along the entire forearm. That is, all preparations are applied to separate sites near the wrist, mid-forearm and near the elbow to minimize the effects that regional variations in drug absorption may have on the resultant blanching response. Preparations, once loaded into the syringes or decanted into containers for pipetting, are coded by a person not directly involved with the application procedure.

The applied formulations, and demarcating labels, remain on the skin for exactly six hours after formulation application. One arm of each volunteer is exposed to the atmosphere for this period (unoccluded mode) and the other arm has each application site, and applied formulation, covered with strips of impervious tape (Blenderm, 3M, South Africa), representing the occluded mode. The former situation allows water to evaporate from the site of application while the latter traps this moisture and hydrates the stratum corneum. The formulations applied to the unoccluded sites are protected from abrasion by clothing for the contact period by lightly taping (Micropore, 3M, South Africa) a porous perspex guard over the entire forearm area. This allows complete exposure of the sites to the atmosphere while precluding physical contact and accidental removal of the formulation. After six hours the perspex guard, occlusive tape and adhesive labels are gently removed from each arm and residual formulation is washed from the application sites with soap and warm water and the area is patted dry with a towel. Erythema induced around the sites by this removal usually subsides within 30 minutes.

Visual assessment of the blanching response is carried out independently by three trained observers at 7, 8, 9, 10, 12, 14, 16, 18, 28 and 32 hours after preparation application, each observer recording the blanching from each volunteer at every time interval. Standard overhead fluorescent lighting is used throughout to illuminate the arms of the volunteers that are presented horizontally for observation. An arbitrary 0-4 point recording scale is used by each observer to subjectively express the degree of blanching observed at each application site: the value 0 representing no discernible blanching, 4 representing intense blanching over the entire site and the intermediate

values representing grades of blanching between the two extremes *(274)*. Additionally, observers make paired comparisons of predetermined adjacent application sites, directly comparing the pallor produced at one site to that at a neighbouring site *(275)*. The observations for this comparison are recorded as 0 (no blanching at either site), = (equivalent blanching at both sites), < or > (blanching at one site being less or greater than that of the compared site). The expression and statistical analyses of these results are described below. Examination of these results clearly indicates the rate and extent of drug release from each of the formulations and allows a postulation to be made regarding the factors affecting the bioavailability of the corticosteroid. This is especially useful if trials have been planned in such a manner as to investigate the effects of single variables on drug release rates from different preparations.

Computation Of Results

Calculation Of Percentage Total Possible Score

The 0-4 graded response values recorded by each observer are pooled for the purposes of evaluation and are used to generate a blanching profile over the observation period. This profile is usually expressed as the percentage of total possible score (%TPS) for each preparation at each observation time and allows calculation of an area under the curve (AUC) value. The total possible score (TPS) attainable for each formulation is calculated as follows:

$$TPS = B \times O \times S \times V \qquad \text{Equation 16}$$

where B is the maximum blanching score attainable at any one site (usually 4), O is the number of observers used in the trial (usually 3), S is the number of sites to which each preparation is applied on each arm (usually 3 when four preparations are simultaneously evaluated) and V is the number of volunteers used in the trial (usually 12). The actual score (AS) for each preparation at each observation interval equals the sum of the graded scores (0-4) recorded by all the observers for that formulation and, hence, the percentage total possible score is given by:

$$\%TPS = (AS / TPS) \times 100 \qquad \text{Equation 17}$$

The percentage total possible score may then be plotted as a function of time for each formulation, generating the blanching profile.

Calculation Of Area Under The Curve

From the blanching profile, the trapezoidal function is used to calculate the area under the curve between each pair of observation times according to the following:

$$AREA = 0.5\ (\%TPS_1 + \%TPS_2) \times (T_2 - T_1) \quad \text{Equation 18}$$

where $\%TPS_1$ is the response at observation time 1 (T_1) and $\%TPS_2$ is the response at the later observation time 2 (T_2). The summation of these individually calculated areas gives the total AUC value for the preparation.

Statistical Analyses

The difference between the observations for any two formulations is statistically assessed by chi-squared analysis. For the graded response observations (0-4 values) a two-by-five contingency table (four degrees of freedom) is constructed to compare the responses of the two preparations at each time interval. Calculated chi-squared values greater than 9.49 indicate significantly different responses at the 95% level.

A two-by-one contingency table is constructed (one degree of freedom) for the paired comparison observations in which only the < and > frequencies are included. Here again, the significance of the difference in the frequencies of <'s and >'s recorded for the comparison of blanching generated by two preparations applied to neighbouring sites is assessed by chi-squared analysis, values greater than 3.84 denote statistically significant difference at the 95% level. In both tests, chi-squared results greater than the datum values stated indicate that one preparation induces statistically superior blanching compared to the other and, therefore, may be assumed to have statistically superior bioavailability.

Provided this methodology is adhered to, the human blanching assay is proven to be sensitive, accurate and reproducible *(53)*. It is an extremely useful tool for assessing the potency and bioavailability of topical corticosteroid preparations and is, therefore, highly applicable to testing extemporaneously prepared formulations. It is advantageous in that the skin of healthy volunteers is used thus obviating the need to locate bilateral dermatological lesions for clinical trials, and, furthermore, several preparations may be tested simultaneously. Compared to other methods of corticosteroid evaluation that have been used *(276)*, the blanching assay is

2. The ability to enter data in any order of observer, volunteer or time interval was also considered of benefit. This would allow data input whenever the operator had spare time as no minimum quantity had to be entered at any one sitting, as was the case with the initial programme using transient computer memory. This may also allow data to be entered between observation times during the process of a trial and the final data analysis could then be available soon after the last observations are made.
3. The facility for checking data input and correction of the same was considered imperative. It was assumed that many of the operators who would use the programme would not be practiced in computer usage and their propensity for error generation would need to be offset by some validation step after data input, prior to permanent storage of this information; a facet that was lacking in the old programme.
4. The ability to carry out all the sorting, calculations and statistical analyses once the blanching data had been entered without further intervention by the operator. The old programme required the results from the preliminary sorting programme to be entered into subsequent statistical programmes for further evaluation, again generating sources of error and adding to the manipulation time.
5. Similarly, the programme should be as "user-friendly" as possible, delivering sufficient information and explanation for completion of the required data input without the need for lengthy vocal instruction by more experienced operators.

With these objectives in mind, a new software package was developed for the Apple II-Plus computer which would carry out as many of the data manipulations, calculations and analyses without the necessity for data re-entry or further operator intervention. The main programme of the package, affectionately named "Superblanch", is written in Applesoft Basic language (Apple DOS 3.3 format) and is listed in Appendix 2. The main programme consists of three sections that are user-selected from the main menu which appears on booting the disk.

Section one must be completed before any raw data from the trial may be stored. This section requires input of all the logistical information for the trial methodology: numbers of volunteers, observers, preparations, application charts, sites per chart and comparisons used in the assay. The name of the trial is requested for initialization of a separate data disk along with the names of the preparations used. All the information entered is permanently stored on the initialized data disk. Most important in this section is the

relatively painless for the volunteers and does not require invasive compromise of the stratum corneum. Moreover, the very close correlation of the blanching response with clinical efficacy of the topical preparation has been established by several studies *(273)*. The method is therefore not only useful but totally appropriate for corticosteroid testing.

COMPUTER ANALYSIS

Initially the data recorded at observation times during the blanching trials was processed manually. The frequency of each response was individually counted on the recording sheets and systematically apportioned using the relevant codes for the preparation, volunteer, application pattern and preparation assignment within each pattern.

To assist in this extremely laborious task a computer programme was written for the Wang 2200 Basic Desk-Top Mini-Computer (Wang Laboratories, U.S.A.) and was later converted for use on the Apple II-Plus Personal Computer (Apple Computer Inc., U.S.A.). This software required the data for each time interval to be entered separately, along with the codes for the application patterns, preparation assignments, etc. The latter information therefore had to be entered on at least 20 occasions as each time interval, occluded and unoccluded modes, was processed, usually more frequently if mistakes were made in the data entry as there were no facilities for error recovery. Re-evaluation of any time interval required re-entry of all the data for that period and it is assumed the potential for undetected errors in this repetitive work would be significant.

Thus, while the initial computer usage was a great improvement over manual counting of the data, there was still scope for improvement in the software which would further alleviate the tedium, and would probably carry out the computations with greater accuracy. A number of facets of the initial programme were considered worthy of improvement:

1. The transfer of the main data handling function from transient computer memory to permanent memory by raw data storage onto a floppy disk. This would require that all the information would be entered into the computer via the keyboard once only. The information would be stored in specific locations on the disk for subsequent reference by the running programme whenever it was needed. This process in itself would save a great deal of hands-on time and would allow re-evaluation of information without the need for data re-entry. The facile evaluation of different permutations of observer and volunteer data would also then be possible.

input of the coding used in assigning letters to designate the preparations, the coding for the preparation layout for each application chart used and the numbers representing sites to be compared for each chart used. This information will be used in the raw data sorting process when the programme deciphers exactly which preparation was responsible for generation of the particular degree of blanching noted at each site using each of the four application patterns. After each piece of information is entered the software requests the operator to check the data before it is permanently saved, if there has been an error in the entry the operator may correct it at this point and the validation step is again effected by the software. In this manner it is very unlikely that an error in the logistical information would be entered by the operator. Furthermore, as this information only has to be entered once, a little extra care and attention at this stage in checking data entry is well worth the time saved when compared to the repetitive, old software. Finally, this section requests the number of observation intervals employed and their times after preparation application. Files are then opened on the data disk and space is allocated to accomodate the raw data for each observer, each volunteer and each time interval in preparation for data input.

Section two of the main programme deals with the storage of the raw data on to the data disk. This requires the graded response and paired comparison data for each time interval, each arm and each observer to be entered separately, however the data may be entered in any order of these permutations. Once the data values are typed into the computer there is, again, a checking step for verification of this information prior to disk storage. However, if an error is subsequently noticed by the operator, the data values stored on the disk may be edited. Here again the correction facilities should preclude the generation of errors in data input. This process takes approximately six hours for a reasonably skilled operator to enter data for three observers and 12 volunteers, compared to double this time for the old programme. A brief help subsection is also available for reference by operators who are not confident with the data input mode.

The real time saving facility of the new programme is only evident in section three which deals with the data sorting and statistical analysis. Prior to these computations the software will check all the raw data entered to ensure there is no data missing and that the application patterns for each volunteer correlate. The remainder of the manipulations requires no operator intervention. The software will sort and count the graded response values and the comparison data for each time interval, this will be used in computation of the %TPS and AUC values for each preparation. Chi-squared statistical

analysis will automatically be carried out on all the graded response and comparison data, and, finally, a dot-matrix print-out of all the data is generated. Additionally, with specific printers, a rough graph of the results (%TPS *versus* time) may be generated. This sorting process takes approximately 60 minutes for three observers and 12 volunteers. This assimilation time is relatively lengthy and is mainly due to the frequent interaction of the software with the data stored on disk: the access time for this information is exceptionally long in comparison to the time required for calculations using the data. Scope for further improvement and acceleration of the programme is, therefore, highly feasible and is currently underway.

A further major improvement with the new programme is the ability to re-evaluate results without the need to re-enter the raw data. Section three requests the operator to enter the specific observers and the specific volunteers to be used in the subsequent sorting and statistical evaluation of data. This facility permits the evaluation of the data from any subgroup permutation taken from the observers and volunteers who participated in the assay. Specific trends in the observer data or response by volunteers may, thus, be easily investigated.

The remainder of the new software package comprises a number of ancillary programmes that are called upon by the main programme to carry out specific functions. There is a greeting programme for the initialization of the data disk, programmes that will carry out the screen plotting and printing of the data as graphs, an evaluation subprogramme which processes the data from both arms of each volunteer together (for use when both arms are occluded for example) and, finally, a programme that will allow raw data to be entered into section two by use of a voice input module. It was initially believed that the latter facility would further diminish the tedium of data entry but experience has shown this method to be more time consuming than simple keyboard entry, probably because the interpretation by the computer of every spoken word must individually be checked. However, this facility is available and may be of use if an operator becomes accustomed to using it.

As with all software, these programmes have not been developed entirely without fault. Blanching assays that have been abnormal, in the use of more than 20 volunteers or the use of only one arm of each volunteer for example, have caused the programme to abort or to generate %TPS values of over 100%. However, these problems are rapidly rectified and the programme is fairly easily modified to handle almost any trial situation. Generally, Superblanch represents a vast improvement over the previous software, not only in the

hands-on time saved by the operator by not having to repetitively enter the same data and manually transfer the results from one programme to another, but also in the verification of all data entered, thereby minimizing error generation. Furthermore, the facility to easily re-evaluate any combination of the stored data is highly advantageous.

Future improvements planned for the programme include the down-loading of all the raw data in to transient computer memory, all calculations can then proceed at the speed of the microprocessor without the need for disk access, a factor which should markedly increase the speed at which data is manipulated. The development of a card-reader facility for the raw data entry would obviate the need for typing the data into the programme via the keyboard. At each observation time the recordings are pencilled directly onto specially designed cards which are subsequently fed into the card reader. The data would instantly be read from the cards, digitized and stored. It would be extremely easy to have the computer read all the data cards between blanching observation times and, with the improved calculation speed, the final results of the trial could possibly be printed minutes after the last observations are made. These are but two modifications that have been planned, as the facilities are used to a greater extent further improvements or additional features will manifest themselves which may then be incorporated.

CORRELATION OF THE BLANCHING ASSAY WITH *IN VITRO* RESULTS

In essence, both the blanching assay and *in vitro* diffusion experiments measure the rate and extent of corticosteroid absorption by the skin. It should, therefore, theoretically be possible to closely correlate findings from both experimental techniques if the same donor preparations are used and *in vitro* methodology has been optimized. Obviously the test of a good *in vitro* diffusion simulation would be the concurrence of its results with those from proven *in vivo* assays. The results from blanching trials have been shown to be highly reliable and one may therefore infer that nonagreement of results may be indicative of poor *in vitro* protocol. Reference to reported literature in this regard demonstrates a wide gradation of *in vitro/in vivo* correlation from total agreement to marked variance.

A number of comparative experiments have been conducted using betamethasone 17-valerate. Busse *et al. (51)* have used two membraneless *in vitro* models to assess the effect of hydrogenated lanolin on the release of the corticosteroid from an ointment base. They found the presence of the lanolin increased the solubility of the drug in the vehicle and improved its delivery

times greater than that of fluocinolone acetonide while their study shows the solubility of fluocinonide to be minimal in the 30% propylene glycol receptor phase used. It is therefore proposed that their *in vitro* results stem more from problems of drug partitioning into nonideal receptor phases than intrinsic diffusivity of the drugs. This situation reiterates the proviso that the *in vitro* diffusion system must be optimized for valid interpolation with *in vivo* data.

From these experiments it appears highly feasible that valid correlations between laboratory diffusion studies and vasoconstrictor assays may be attainable with meticulous planning and execution of the methodology. It has been shown, using elementary experiments without barrier media, that the rate of release of a drug from dissimilar topical vehicles follows rank order agreement with the blanching responses elicited by these formulations. Similar results have been demonstrated using more elaborate laboratory diffusion apparatus. However, in most of the cases reported the dissimilarity between the formulations tested has been vast. For example, the inclusion of hydrogenated lanolin by Busse *et at. (51)* improved the solubility of the corticosteroid several fold in the vehicle they tested. Vastly improved release is, therefore, expected and may be demonstrated even by the crudest of laboratory methodology. Similarly, increasing the proportion of propylene glycol in a gel formulation changes the environment of the drug from one of minimal solubility, to one of optimal solubilization and finally produces a situation of over solubilization with concomitant decrease in the partitioning potential between vehicle and skin. Here, *in vitro* techniques would easily demonstrate poor release, optimal release and declining release characteristics, respectively.

It is, therefore, proposed that the mark of a good *in vitro* diffusion cell system would be more accurately indicated by its ability to distinguish between the permeation of drug from very similar donor formulations, for example, different commercial preparations of the same dosage form containing the same concentration of drug. It is assumed that each manufacturer optimizes the vehicle formulation to demonstrate the best release characteristics for the drug and, thus, the *in vitro* system used would have to be extremely sensitive to distinguish between the very subtle differences in the products tested. It is doubtful whether Petri dishes packed with formulation and immersed in receptor fluid would be able to accomplish this distinction. Fairly complex diffusion systems are therefore required with optimized hydrodynamic and partitioning characteristics, and would probably require the use of biological tissue for the diffusion medium if the extremely complex process of percutaneous absorption is to be simulated closely. These factors

White Soft Paraffin	13.50 g
Propylene Glycol	10.50 ml
Cetostearyl Alcohol	7.01 g
Liquid Paraffin	5.40 g
Citric Acid	0.89 g
Sodium Lauryl Sulphate	0.78 g
Disodium Hydrogen Phosphate	0.65 g
Betamethasone 17-Valerate	0.12 g
Chlorocresol	0.09 g
Water q.s. ad	100.0 g

Batches of the base were also compounded to include:

Urea	10%
Resorcinol	2% or 5%

The aqueous fraction was heated to 60 °C and slowly added to the lipid fraction in a prewarmed, tared, mortar with constant stirring. When the mixture had cooled and solidified slightly (approximately 40 °C) the corticosteroid solution was added and its vial rinsed with further aliquots of warm water to ensure complete content transfer. The mass of the mixture was then adjusted to 100 g with water and the formulation triturated vigorously until cold (15 to 20 minutes) using a pestle. If meticulous attention is paid in this manner to the temperatures of the constituents and prewarmed apparatus is used a very smooth, white, fine emulsion may be produced which does not separate, even after several years of storage. The prepared formulations were packed into screw-cap ointment jars and stored in the dark at room temperature.

Seven blanching trials were conducted in total to evaluate the commercial and extemporaneous betamethasone 17-valerate preparations and the two hydrocortisone products. Table X details the products tested in each trial and the application modes used.

TABLE X: Summary of blanching trials conducted.

Trial	Formulation	Products Tested	Application Mode
1	Cream	B C P	Unocc
2	Lotion	B C	Unocc
3	Ointment	B C P	Unocc
4	All*	B	Occ/Unocc
5	Cream	EXT-R	Occ/Unocc
6	Cream	EXT-U	Occ/Unocc
7	Cream	H L	Occ

EXT-U and EXT-R extemporaneous preparations containing urea and resorcinol, B Betnovate, C Celestoderm-V, P Persivate, H Cutaderm, L Lennon 0.5% hydrocortisone.
* Cream, ointment, lotion and scalp application.

have been borne in mind in the design, planning and attention to detail of the present *in vitro* diffusion cell with the aspiration of possibly producing a laboratory system representative of the absorption which occurs *in vivo*.

PREPARATIONS EVALUATED USING THE BLANCHING TRIAL

The blanching trial methodology described above was used to evaluate the bioavailability of a number of proprietary and extemporaneously prepared betamethasone 17-valerate containing topical formulations. The formulations tested comprised the full range of commercial products containing this corticosteroid available on the South African market and a laboratory-prepared formulation selected for optimal drug stability. The commercial products tested were Betnovate cream, ointment, lotion and scalp application (Glaxo, South Africa); Celestoderm-V cream, ointment and lotion (Scherag, South Africa) and Persivate cream and ointment (Lennon, South Africa). In addition, two hydrocortisone containing creams were evaluated each containing the drug at 0.5% concentration: Cutaderm (Scherag, South Africa) and an experimental formulation (Lennon, South Africa). Assay values for each preparation are given in Appendix 1.

The stability studies of Bundgaard and Hansen *(277)* have indicated betamethasone 17-valerate to be most stable to C-17 ester rearrangement degradation at pH3.5. A modified buffered cream formulation *(278)* was chosen for the extemporaneous vehicle base and the citric acid-disodium hydrogen phosphate buffer system was adjusted to yield a cream with an apparent pH of approximately this value. The prepared creams were formulated to contain various concentrations of either urea (0% or 10%) or resorcinol (0%, 2% or 5%) to test for their penetration enhancing effects on the steroid. The preparations were compounded (as detailed below) and assayed (as detailed in section 3.1) on the day prior to the blanching trial so that the effects of any possible aqueous degradation of the steroid due to aging would be minimized. The formula employed for the cream base is listed below and produced an emulsion with a final betamethasone concentration of 0.1% (equivalent to that of the commercial preparations).

The white soft paraffin, cetostearyl alcohol and liquid paraffin were melted together in an evaporating dish over a water bath and maintained at 60 60 °C. Citric acid, sodium lauryl sulphate, disodium hydrogen phosphate and chlorocresol were dissolved in approximately 30 ml of warm water along with the urea or resorcinol where appropriate. The betamethasone 17-valerate was dissolved in the propylene glycol fraction with the aid of ultrasonification.

into both chloroform and isopropyl myristate-impregnated filter paper. A similar enhancement of the blanching response was noted in the presence of the lanolin. Amundsen *et al. (57)* have used a Petri dish filled with gel to measure the *in vitro* release of betamethasone 17-valerate into isopropyl myristate. They found that the release and blanching response decreased as the percentage of propylene glycol in the gel increased from 30% to 90%. Washitake and coworkers *(93)* have compared the vasoconstrictor response of this steroid contained in various ointment vehicles with its permeation through egg shell, cellulose and polyamide lipoid membranes. They found fair rank order agreement between the mass of drug permeating and blanching response except for a macrogol ointment which exhibited exceptionally high diffusivity *in vitro* but poor *in vivo* response. This result is probably due to the *in vitro* experimental technique using the nondiscriminatory membranes which allowed water to diffuse into the donor chamber and dissolve the vehicle.

Barry and Woodford *(211)* have shown that the decreasing blanching response to serial dilutions of betamethasone 17-benzoate gel is paralleled by decreasing release rates *in vitro* using a cellulose bag to contain the gel. Malone *et al. (72)* have used a simple teflon cell with non rate-limiting filter paper membrane to evaluate the effect of solubility on the release of fluclorolone acetonide from cream and ointment formulations. *In vitro* release and *in vivo* blanching were shown to be greater for the formulations in which the drug was presented in saturated solution than for the vehicle in which the drug was poorly soluble. Rosvold *et al. (56)* found that release of the same corticosteroid from gels into isopropyl myristate decreased as the propylene glycol concentration in the gel increased, however, the blanching potential of the vehicles was found to be less dependent on solubilizer content.

More elaborate *in vitro* diffusion cells containing excised human skin as the diffusion medium have been used by several workers. Katz and Poulsen *(55)* have reported greater blanching and higher *in vitro* release for fluocin-olone acetonide completely solubilized in propylene glycol. Poulsen *(54)* has reported similar results for fluocinolide. Ostrenga *et al. (139)* and Haleblain *et al. (53)* have carried out similar investigations with fluocin-olone acetonide and fluocinonide in various propylene glycol concentrations. In all cases maximum blanching and *in vitro* release were observed when the cosolvent was present only in sufficient concentration to completely solubilize the permeant. Ostrenga *et al.* found the permeation of fluocinolone acetonide *in vitro* to be two-thirds greater than that of fluocinonide although the latter drug was found to elicit a greater blanching response. The authors explain this by assuming the intrinsic potency of fluocinonide to be three

The methodology for trial four varied slightly from that described above. In this assay the blanching response to equivalent masses of betamethasone 17-valerate presented in Betnovate cream, ointment, lotion and scalp application dosage forms was tested. Initially, the products were each analyzed to determine their exact drug concentration per unit mass. Thereafter, the quantity of each formulation to be applied to the arm was calculated so that each site would be exposed to exactly the same mass of corticosteroid, but contained within differing vehicle volumes. In this manner the effect of the formulation vehicle on the bioavailability of the drug could accurately be assessed as the mass of steroid applied to each site would be uniform throughout. The only factor, therefore, affecting the degree of blanching generated would be the release and partitioning characteristics of the vehicle. It was believed this assay would be useful in the investigation of vehicle effects on drug absorption and would augment results from the *in vitro* diffusion cell system. In all cases the results from the blanching trials were compared to those from the *in vitro* investigations and correlations between the two groups assessed. These results are presented in Section 4.

4. RESULTS AND DISCUSSION OF PERMEATION AND BLANCHING EXPERIMENTS

On the basis of the diffusion experiment methodology, the high-performance liquid chromatography and blanching assay techniques described previously, the following results are presented for the permeation investigations undertaken. In all cases the *in vitro* results, tabulated or plotted graphically, are of the mean, cumulative, total mass of corticosteroid that has permeated through unit area of membrane with respect to the time after initiation of the experiment, and are reported in terms of $\mu g\ cm^2$ units. These masses are calculated from the assay values of the receptor chamber drug concentration of at least three replicate experiments, corrected for previous sample aliquots removed and corrected for the diffusion area of the membrane. The standard deviations (SD) of the reported mean values are also presented in the tables (usually in parentheses) and are represented graphically by a vertical line through the mean point. The statistical significance of the difference between mean permeation masses from different experiments has been assessed using the Student's t distribution test (null hypothesis of equal means—variance unknown) *(279)*, and, where appropriate, results are simply reported as being significantly different at the 95% level of confidence. Special comment is made on the statistical evaluation where this is deemed appropriate. The results and statistical evaluation of the blanching trial results are reported as detailed in Section 3.6.

4.1 RESULTS OF DIFFUSION CELL DESIGN VALIDATION

AGITATION

Valid performance of an *in vitro* permeation cell system requires that diffusion boundary layers at the membrane interface are minimized by adequate agitation of the receptor chamber fluid. Only in the presence of satisfactory hydrodynamic mixing within the cell will partitioned molecules of permeant be swept away from the membrane surface, maintaining sink conditions as the driving force for further diffusion. Furthermore, if diffusion boundary layers are established, they may contribute significantly to the overall resistance to permeation, these layers representing barriers in series to the mass transfer of the drug. In these situations low, misleading permeation rates may be measured from which erroneous conclusions may be drawn. Initial

diffusion cell validation, therefore, necessitates the demonstration that the applied agitation is of sufficient intensity to minimize diffusion boundary layers and, thereby, optimize the drug permeation rates. Characterization of the contribution that the stagnant diffusion layers make to permeation resistance has been accomplished by empirically determining the limiting magnetic bar stirring speed above which further increases in the revolution rate produced no measurable change in the permeability of the membrane.

The effect of receptor fluid agitation on the measured permeation rate of betamethasone 17-valerate from a 0.1% solution of this drug in isopropyl myristate to purified isopropyl myristate receptor phase, through silicone membrane, was assessed using the steady-state diffusion cell configuration as described in Section 3.5. Differential agitation rates were generated by varying the revolution speed of the laboratory stirrer, which drove the teflon bar magnets in the cell receptor chambers, between 0 rpm and 1150 rpm at 200 rpm intervals. The average corticosteroid masses permeating into the receptor chambers with respect to time at these different stirring speeds are reported in Table XI. All experiments were carried out at 35 °C.

TABLE XI: Mean drug mass permeating unit silicone membrane area at different stirrer revolution speeds & 35 °C. ($\mu g\ cm^{-2}$).

Time (hours)	Stirrer-bar revolution speeds (rpm)						
	0	200	400	600	800	1000	1150
0.5	5.37	5.86	7.65	8.66	9.36	10.30	10.83
1.0	11.26	14.00	18.69	19.71	19.92	21.86	21.01
1.5	16.89	18.66	29.88	31.44	28.50	33.37	31.49
2.5	28.15	35.78	51.11	53.65	49.10	55.74	53.07
4.0	45.04	57.10	85.68	91.87	82.03	91.37	87.08
6.0	67.56	91.25	131.89	137.92	124.94	136.63	129.57
9.0	101.34	131.34	208.03	199.41	195.69	209.87	199.18
12.0	135.12	180.09	271.56	266.06	258.53	270.84	258.40
CC	0.9986	0.9996	0.9997	0.9996	0.9998	0.9998	0.9998

CC Correlation coefficient.

The rate of corticosteroid permeation increases as the agitation of the receptor cell fluid is increased from the static situation (0 rpm). It is evident that the rate increases markedly between 0 rpm and 400 rpm while at stirring speeds greater than the latter value no further, significant increase in the drug permeation rate is evident. This would suggest that the plateau level, representing optimal fluid mixing and optimal dissipation of the diffusion boundary layer at the membrane interface, is achieved at approximately 400rpm. It is appropriate, therefore, to combine the masses measured at stirring speeds between 400-1150 rpm into one composite mean and report these values with respect to the permeant masses at the nonoptimal (0 rpm and 200 rpm) stirring conditions *(24,40)*, this data is presented in Table XII.

Figure 23 depicts the linear least squares regression plots of the composite data from Table XII. The relatively small standard deviations of

the composite 400-1150 rpm means imply that the summation of this data is a valid procedure and that these stirring speeds represent optimal agitation conditions. Statistically, the 400-1150 rpm flux values are significantly different to the 0 rpm and 200 rpm values at all sampling times. The 200 rpm values are significantly different to the 0 rpm values at sampling times after 2.5 hours, all comparisons at the 95% level of significance.

TABLE XII: Comparison of mean drug masses permeating unit silicone membrane area at optimal and non-optimal stirrer revolution speeds & 35 °C. (μg cm-2).

Time (hours)	Stirrer bar revolution speeds (rpm) 0	200	400 - 1150
0.5	5.37 (1.61)	5.86 (0.32)	9.36 (1.28)
1.0	11.26 (0.65)	13.99 (0.79)	20.24 (1.22)
1.5	16.89 (0.97)	18.66 (1.05)	30.94 (1.84)
2.5	28.15 (1.62)	35.78 (1.27)	52.53 (2.53)
4.0	45.04 (2.59)	57.10 (0.82)	87.61 (4.11)
6.0	67.56 (3.89)	91.25 (0.95)	132.19 (5.29)
9.0	111.31 (5.84)	131.34 (4.02)	202.44 (6.16)
12.0	148.41 (7.78)	180.09 (5.32)	265.08 (6.40)
CC	0.9989	0.9996	0.9991

Standard deviations of means in parentheses.
CC Correlation coefficient.

TABLE XIII: Average, apparent drug permeability coefficients for silicone membrane, calculated at different stirrer bar revolution speeds & 35 °C.

Revolution speed (rpm)	Permeability coefficient ($cm\ h^{-1} \times 10^2$)	SD
0	1.154	0.051
200	1.394	0.123
400	2.159	0.124
600	2.212	0.081
800	2.054	0.107
1000	2.267	0.040
1150	2.147	0.045
400-1150	2.148	0.126

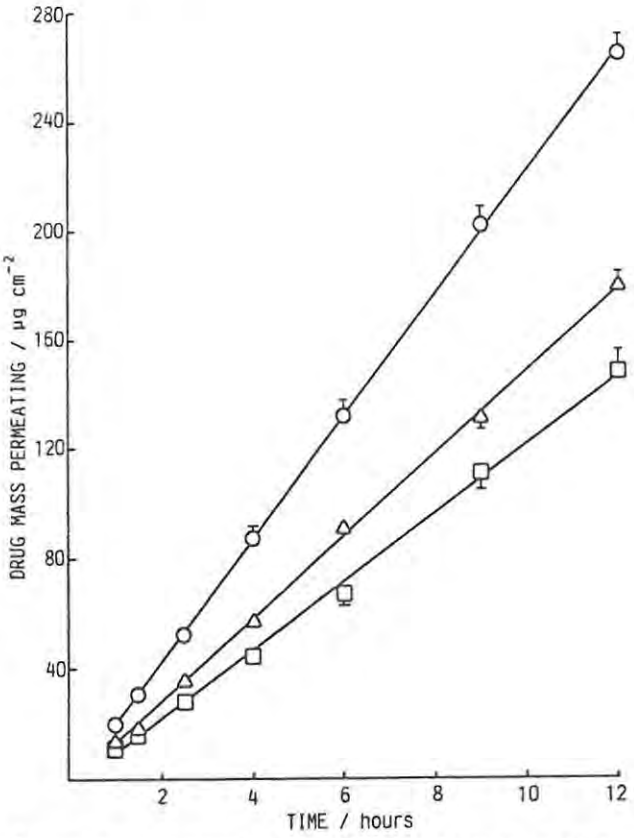


FIGURE 23: Permeation of betamethasone 17-valerate through silicone membrane at 35 °C and different chamber agitation speeds. □ 0 rpm, △ 200 rpm, ○ 400-1150 rpm.

It is interesting to examine the effect of stirring speed on the apparent permeability coefficient of betamethasone 17-valerate passing through silicone membrane, its interfaces and the stagnant diffusion layers on either side of the membrane at 35 °C *(44,97)*. The apparent permeability coefficient of the

drug at each sampling interval may be calculated by the quotient of the flux rate and donor concentration (Equation 8). The average coefficient value at each stirring speed is listed in Table XIII. These values indicate the relatively constant, plateau permeability coefficient values attained at stirring speeds greater than 400 rpm. The values are represented graphically (Figure 24) as a plot of the permeability coefficients *versus* the inverse square root of the stirring speed. The constancy of the permeability coefficient at higher stirring speeds is clearly apparent from this representation. This graph may also be used to estimate the permeability coefficient in effect at infinite stirring speed where, theoretically, there would be no stagnant diffusion layers present on either side of the membrane. This is accomplished by extrapolating the plateau, linear portion of the graph to the ordinate (where stirring speed is infinite and $rpm^{-\frac{1}{2}} = 0$). Estimation of the extrapolated permeability coefficient in this manner yields a value of 2.1645×10^{-2} cm h^{-1} (SD = 0.07154×10^{-2} cm h^{-1}).

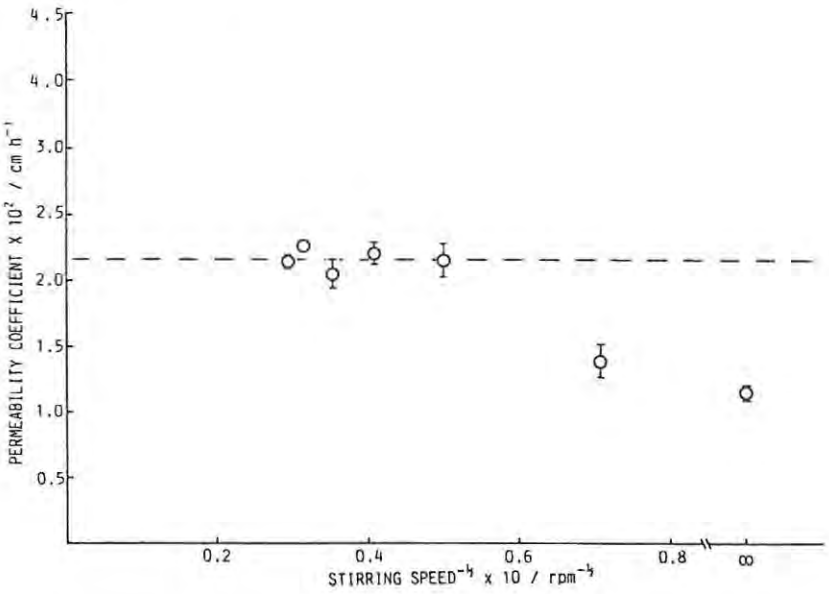


FIGURE 24: Effect of agitation speed on the permeability coefficient of betamethasone 17-valerate traversing silicone membrane at 35 °C.

It is therefore apparent that the diffusion cell system performs as anticipated with respect to degree if internal fluid agitation. The design has been validated and proven with regard to its capability of maximally dissipating the diffusion boundary layers at the receptor surface of the membrane. The system may, therefore, be used with confidence to predict the *in vitro* permeation of betamethasone 17-valerate. It is noteworthy that optimal hydrodynamic mixing is achieved at stirring speeds as low as 400 rpm, at the lower range of the agitation capabilities of the design. This

observation corroborates the theory underlying the planning of this new cell conformation and substantiates the belief that this design would exhibit good hydrodynamic mass transfer. It is also interesting to note the high levels of drug detected in the case where the receptor solution was not stirred at all, the kinetic energy of the permeant molecules at 35 °C obviously aiding drug diffusion through the receptor phase. Kinetic energy of this nature would obviously be of benefit in nonideal cell designs by assisting mass transfer, especially with aqueous receptor phases.

On the basis of these results, a stirring speed of 600 rpm was chosen for all further experiments as a value well within the optimal permeation range of the drug yet not so vigorous as to impose undue strain on the magnetic stirrers or system as a whole.

TEMPERATURE

Complete validation of the new *in vitro* diffusion cell design also required an investigation into the effect of temperature on the permeation rate of betamethasone 17-valerate through various media. It is imperative that the system exhibits sensitivity to ambient temperatures so that performance would parallel that of other cell designs reported in the literature. In this regard, infinite dose, steady-state permeation experiments were carried out using a 0.1% solution of the drug in isopropyl myristate as the donor phase and purified isopropyl myristate as the receptor solvent. Agitation rate was fixed throughout at 600 rpm and the water bath temperature, into which the cells were immersed, was precisely controlled (+ 0.5 °C) at different values between 20 °C and 40 °C. Silicone membrane was chosen as a model synthetic medium for these studies and, for comparison, the effect of temperature on the permeation through human and weanling pig stratum corneum, and full thickness hairless mouse skin was also investigated. The cumulative masses of corticosteroid permeating unit area of silicone membrane with respect to time at 600 rpm agitation and various temperatures are listed in Table XIV.

TABLE XIV: Mean drug mass permeating unit silicone membrane area at different temperatures and 600 rpm agitation. ($\mu g\ cm^{-2}$).

Time (hours)	Temperature (°C)				
	20	25	30	35	40
0.5	3.12(0.39)	4.20(0.37)	6.34(0.86)	8.66(0.76)	10.86(2.67)
1.0	8.38(0.91)	9.68(0.56)	14.45(1.39)	19.71(0.08)	22.68(0.67)
1.5	12.78(1.09)	15.85(1.13)	21.62(1.34)	31.44(0.08)	34.04(0.86)
2.5	23.28(3.22)	28.81(0.97)	36.99(1.80)	53.65(0.67)	59.74(1.66)
4.0	36.31(5.49)	46.69(1.65)	58.68(1.95)	91.87(0.65)	97.50(2.25)
6.0	55.36(4.47)	73.22(4.44)	88.65(3.06)	137.92(0.37)	139.62(2.73)
9.0	91.22(8.56)	109.75(5.27)	135.54(7.97)	199.41(4.78)	229.83(2.01)
12.0	118.39(9.42)	148.27(5.99)	179.17(7.50)	266.06(0.71)	287.87(2.11)
CC	0.9992	0.9999	0.9999	0.9996	0.9991

Standard deviations of means in parentheses.
CC Correlation coefficient.

These values are plotted in Figure 25 which illustrates the increase in drug permeation rate observed with increase in temperature. These observations are in congruity with those reported by other researchers *(13,97,107, 120,124)* and are explained in terms of the kinetic energy of the permeant molecules. At higher temperatures the energy exhibited by each molecule is relatively greater which augments the random movement in the direction of the concentration gradient. Higher kinetic energy will also increase the transience of bonding interactions between permeating molecules and polymeric groups within the membrane, thereby also decreasing the transit time between the interfaces of the membrane. Statistically, only neighbouring values at the 0.5-hour sampling time and the 20 °C and 25 °C values at 1.0 hour are insignificantly different, all other values exhibit significant difference to neighbouring values at the same sampling time.

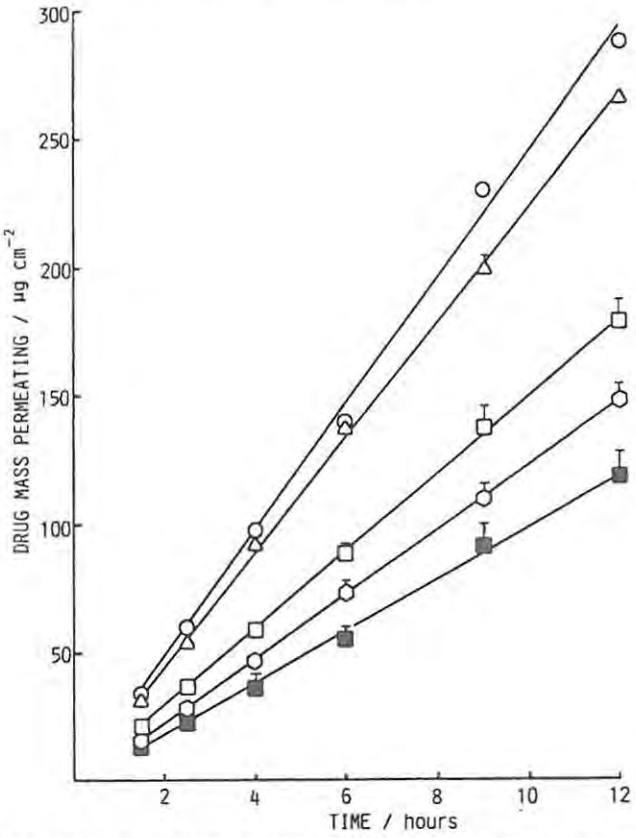


FIGURE 25: Permeation of betamethasone 17-valerate through silicone membrane at 600 rpm agitation and different chamber temperatures. ■ 20 °C, ○ 25 °C, □ 30 °C, △ 35 °C, ○ 40 °C.

Noteworthy from this graph is the good precision of the data values: at each sampling time the variance in the mass of permeating drug is relatively small. This diffusive uniformity would be expected from a synthetic polymer which, because of its controlled manufacturing process, would present an identical diffusive barrier in any number of replicate cells. It is therefore assumed that analytical and sampling techniques would represent the main sources of the error when monitoring permeation through silicone membrane.

In contrast, the cumulative masses of corticosteroid permeating unit area of human stratum corneum, weanling pig stratum corneum and full thickness hairless mouse skin with respect to time at 600 rpm agitation and various temperatures are presented in Tables XV-XVII, and these data are plotted in Figures 26-28, respectively.

These graphs are typical drug permeation profiles exhibiting an initial, nonlinear segment followed by a steady-state portion. As with the silicone membrane, the permeation rates through all three animal media appear proportional to the experimental temperature. Immediately apparent from this data is the large difference in the magnitude of the flux through the synthetic and animal media.

TABLE XV: Mean drug mass permeating unit area of human stratum corneum membrane at different temperatures and 600 rpm agitation. ($\mu g\ cm^{-2}$).

Time (hours)	Temperature (°C) 30	35	40
16	0.843 (0.073)	1.203 (0.686)	3.083 (0.829)
24	1.381 (0.054)	2.559 (0.891)	5.888 (1.059)
30	2.491 (0.227)	3.756 (0.812)	7.359 (1.324)
40	3.645 (0.554)	5.603 (1.641)	10.899 (4.125)
48	4.407 (1.445)	6.887 (1.308)	12.510 (3.974)
60	6.244 (2.563)	8.572 (2.278)	16.518 (6.173)
72	7.521 (3.222)	11.337 (3.849)	19.222 (7.039)
CC	0.9968	0.9982	0.9985

Standard deviations of means in parentheses.
CC Correlation coefficient.

TABLE XVI: Mean drug mass permeating unit area of weanling pig stratum corneum membrane at different temperatures and 600 rpm agitation. ($\mu g\ cm^{-2}$).

Time (hours)	Temperature (°C) 30	35	40
16	1.520 (0.231)	3.545 (2.215)	7.575 (1.214)
24	1.867 (0.481)	4.886 (3.852)	12.374 (2.746)
36	3.318 (1.382)	11.191 (6.917)	19.408 (1.569)
48	5.314 (1.023)	14.908 (7.476)	27.008 (3.682)
60	6.406 (1.813)	17.749 (6.148)	32.347 (6.951)
72	8.618 (1.506)	21.838 (6.565)	37.121 (5.970)
CC	0.9958	0.9895	0.9964

Standard deviations of means in parentheses.
CC Correlation coefficient.

TABLE XVII: Mean drug mass permeating unit area of full thickness hairless mouse skin at different temperatures and 600 rpm agitation. ($\mu g\ cm^{-2}$).

Time (hours)	Temperature (°C) 30	35	40
4	2.963 (0.306)	4.288 (0.803)	7.104 (1.299)
8	5.822 (0.775)	9.278 (2.037)	16.623 (3.299)
16	11.920 (1.409)	22.961 (3.447)	45.437 (5.486)
24	20.486 (3.211)	37.680 (5.087)	68.591 (3.152)
40	38.998 (5.047)	61.517 (3.694)	110.887 (2.213)
48	48.870 (3.867)	81.620 (2.221)	134.420 (5.624)
64	68.868 (3.312)	110.187 (3.266)	180.458 (12.947)
72	79.027 (5.133)	123.046 (5.482)	209.828 (6.741)
CC	0.9993	0.9988	0.9988

Standard deviations of means in parentheses.
CC Correlation coefficient.

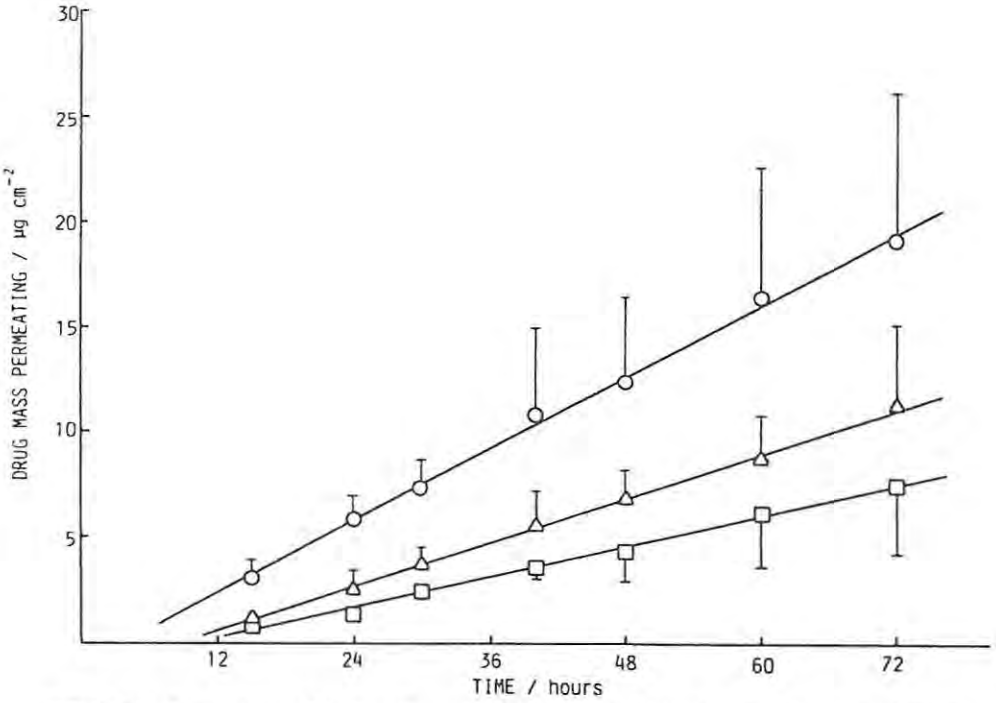


FIGURE 26: Permeation of betamethasone 17-valerate through human stratum corneum at 600 rpm agitation and different chamber temperatures.
□ 30 °C, △ 35 °C, ○ 40 °C.

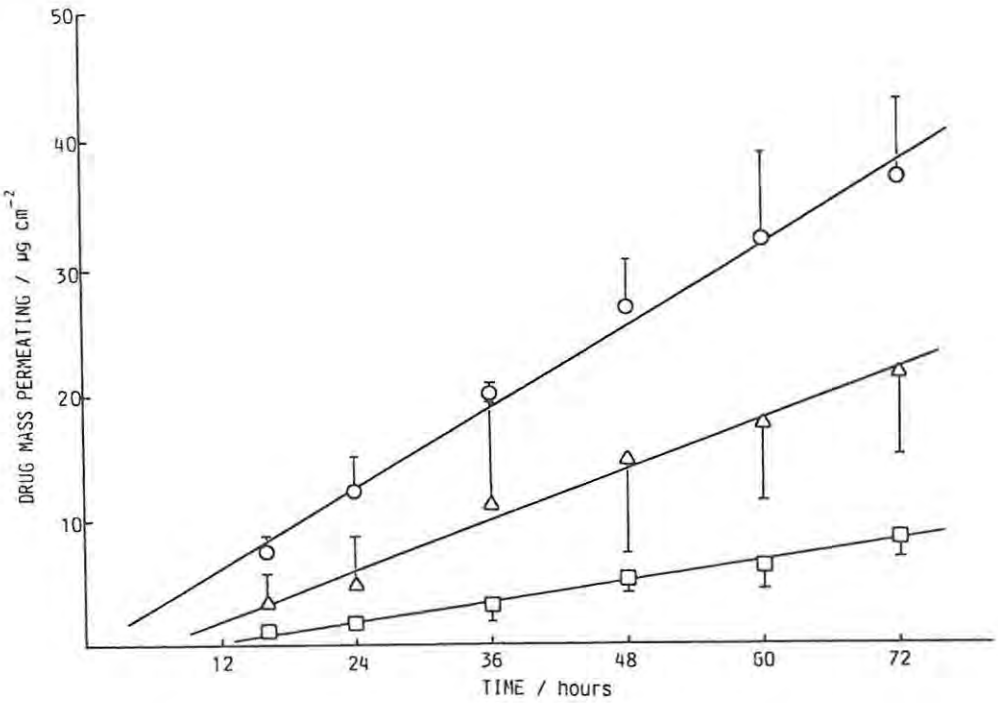


FIGURE 27: Permeation of betamethasone 17-valerate through weanling pig stratum corneum at 600 rpm agitation and different chamber temperatures.
□ 30 °C, △ 35 °C, ○ 40 °C.

The corticosteroid permeation is approximately 300-fold greater through silicone membrane than human stratum corneum, 100-fold greater through weanling tissue and 15-fold greater through hairless mouse skin, per unit time at equivalent temperature. These values exemplify the outstanding barrier

properties of the stratum corneum cells in comparison to synthetic media to the passage of exogenous substances. The rank order of permeability among the animal membranes closely parallels that reported by numerous other researchers *(27,48,78,112,152,180,181,183,184)*: hairless mouse skin is more permeable than weanling stratum corneum, which is slightly more permeable than human tissue. The permeation values measured here through the hairless mouse skin are higher than values reported by Chien and Valia *(115)* for the permeation of estradiol and this is assumed to be due to the higher intrinsic diffusivity of the betamethasone moiety.

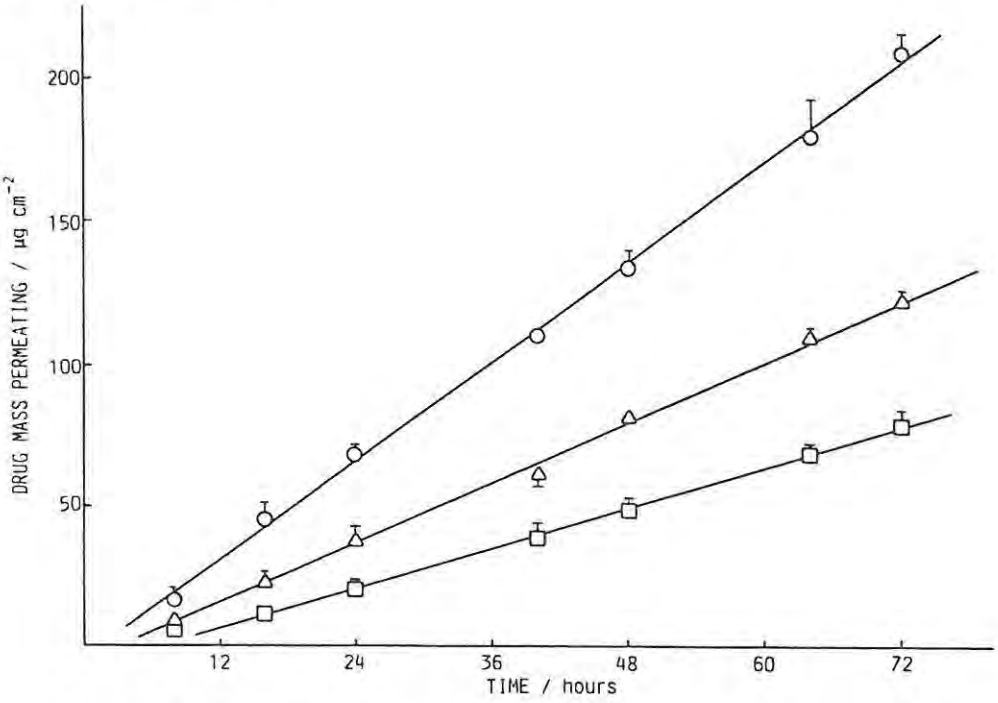


FIGURE 28: Permeation of betamethasone 17-valerate through full thickness hairless mouse skin at 600 rpm agitation and different chamber temperatures. □ 30 °C, △ 35 °C, ○ 40 °C.

Furthermore, the variance in the results from the biological media is markedly more pronounced than that from silicone membrane. At 35 °C the standard deviations of the silicone permeation data approximate 1.8% of the actual drug masses at each sampling time. At the same temperature the standard deviations for the human tissue data average 31.8% of the drug masses. Although variability of this magnitude is well within the range expected for biological tissue *in vitro (2,85)*, the variance is substantially worse with the weanling tissue (53.0%) and significantly better with the hairless mouse skin (10.67%). The relatively high variability of the weanling stratum corneum performance is believed to be a factor of the nonideal method used in sampling and preparation of this membrane (detailed in Sections 3.3 and 3.4). The apparent, relative uniformity of the mouse skin may be due both to the use of non-separated tissue and to the use of a relatively small number

of donor animals. The possibly deleterious effects of attempting to separate the upper skin strata were therefore avoided and the use of a small donor population would tend to contain the variability.

For the mouse skin at each time interval there are statistically significant differences between the permeation data values at neighbouring temperatures. On the other hand, for human tissue at certain time intervals, some of the data points from different temperatures exhibit no significant statistical difference. This is mainly due to the relatively large standard deviations of the mean values that may overlap standard deviations from neighbouring plots. In these cases the statistical test that has been applied indicates that there is a 5%, or greater, probability that the differences in permeation rates observed at the different temperatures are not real but artefactual differences, possibly introduced by the experimental protocol. These instances of statistical indifference between the mean permeation values occur at the 16-, 40-, 60- and 72-hour intervals for the 30 °C and 35 °C plots, and at 40, 60 and 72 hours for the 35 °C and 40 °C data comparison.

A similar trend is exhibited for the weanling stratum corneum, there is no statistically significant difference at 16, 24 and 36 hours for the 30 °C and 35 °C comparison, and at 36 hours for the 35 °C and 40 °C plots. As with all statistical evaluations, these results are open to wide interpretation. It is proposed that the apparent equivalence of several data values, as suggested by the statistical evaluation, should not detract from the observation that higher temperatures generate higher permeation rates, regardless of the membrane used in the study.

As a further validation of the diffusion cell design, it is valuable to examine these data for violation of sink permeation conditions. As there is no total replacement of the receptor medium during the experiment, the possibility exists that permeant mass may increase sufficiently in the receptor chamber to diminish the concentration-differential driving force to further permeation. For these static diffusion cell designs, it is generally accepted that sink diffusion conditions have not been violated provided the permeant mass in the receptor phase does not exceed 10% of the donor phase concentration *(2)*. The concentration of betamethasone 17-valerate in the donor phase was assayed at the initiation and completion of each experiment, the percentage change in donor concentration may thus be assessed. Furthermore, knowing the cumulative amount of drug that has entered the receptor chamber over the course of the experiment, and the amount that has permeated from the donor compartment, an estimation of the extent of drug binding to the membrane (reservoir effect) may be made. These assessments for each of the

animal and silicone membranes are presented in Table XVIII as the percentage change in donor vehicle drug concentration over the course of the experiment, the final receptor phase concentration as a percentage of the donor phase concentration, and the percentage of drug mass permeating from the donor chamber that is retained by the membrane.

TABLE XVIII: Relative permeant disposition in membrane, donor and receptor phases at 35 °C.

Membrane	% change in donor phase concentration	Final receptor concentration as % of donor	% of permeating mass retained by membrane
Silicone	7.83	4.86	37.93
Hairless Mouse	6.81	2.28	66.51
Weanling pig	4.13	0.24	94.21
Human	4.22	0.17	95.88

It can be seen from these values that the 10% receptor concentration datum for violation of sink conditions is not attained, even by the silicone membrane which exhibits the greatest permeability and yields a final receptor concentration approximating 5% of the donor solution strength. The concentrations for the other membranes are significantly lower and are well within the 10% limit. Similarly, the donor solution did not decrease in concentration by more than approximately 8%, again exhibited by the silicone membrane. It can, therefore, be assumed that sink permeation conditions are adequately maintained throughout each experiment.

It is interesting to note the percentage of permeant that leaves the donor chamber but is sequestrated by the membrane in each case. A relatively small percentage is retained by the silicone membrane in comparison to the animal media, as would be expected. Relatively large percentages are bound by the human and weanling horny layers in comparison to the full thickness hairless mouse skin, although the absolute masses of drug in the two cases are of different magnitude and the stratum corneum reservoir values may, thus, be slightly exaggerated by the percentage calculations. It is interesting, also, to speculate on the residency of the drug reservoir in the stratum corneum. The horny layers of the human and pig specimens are able to retain large percentages of the drug entering these media, while the whole skin from the hairless mouse does not exhibit greater reservoir potential in relation to its thickness. This may confirm that the major proportion of drug sequestration occurs in the outermost skin layers.

As previously suggested, an increase in the permeation temperature should increase the kinetic energy of the diffusant molecules within the membrane, thereby accelerating their transit through the medium. These kinetic effects should be evident from the magnitudes of the diffusion coefficients for the drug at different temperatures. One facile method of calculating the diffus-

ion coefficient from permeation profiles such as these is by use of the lag time technique *(13)*. The linear, steady-state portion of each plot may be extrapolated, by least square regression, to the abscissa to obtain an estimation of the lag time. This is an indication of the time required for equilibration of the diffusant within the membrane and, theoretically, this period should be shorter at higher temperatures. Having some estimation of the membrane thickness *(182)*, the diffusion coefficient may be calculated from the quotient of the square of the thickness to six times the lag period (Equation 9). Once the diffusion coefficient has been calculated, the partition coefficient for the drug between membrane and bathing fluid may be calculated by the quotient of the product of permeability coefficient and thickness, to the diffusion coefficient (Equation 10). For comparison these values, calculated at the different temperatures, are presented in Table XIX.

TABLE XIX: Calculated permeation parameters for betamethasone 17-valerate across synthetic and animal membranes between 20-40 °C, and 600 rpm agitation.

Membrane	Temperature (°C)	Lag Time (hours)	Permeability coefficient (cm h^{-1} x 10^4)	Diffusion coefficient (cm h^{-1} x 10^8)	Partition coefficient
Silicone	20	0.2469	88.46	10887.7	1.0319
	25	0.2167	110.74	12405.0	1.1337
	30	0.0640	144.73	41996.0	0.4377
	35	0.0581	221.18	46291.8	0.6068
	40	0.0554	235.56	48540.4	0.6163
(Membrane thickness = 0.0127 cm)					
Human	30	10.0938	0.8353	4.6603	3.0112
stratum	35	8.6525	1.2727	5.4366	3.9329
corneum	40	3.6269	2.5124	12.9698	3.2544
(Stratum corneum thickness = 0.00168 cm)					
Weanling pig	30	11.0770	1.0036	10.4866	2.5266
stratum	35	6.1309	2.7428	18.9467	3.8218
corneum	40	0.5078	5.2424	22.8752	0.6050
(Stratum corneum thickness = 0.00264 cm)					
Hairless	30	6.8327	9.0421	11952.4	0.5296
mouse skin	35	3.1685	14.8823	25774.8	0.4042
	40	1.5002	26.0729	54438.3	0.3353
(Whole skin thickness = 0.07 cm)					
	30			24.9779	11.1584
	35			53.8636	8.8415
	40			113.7399	7.3355
(Effective membrane thickness = 0.0032 cm)*					

* See text for explanation.

The rate of initial equilibration and the steady-state diffusion of the drug will be governed by the structural characteristics of the membrane and the extent of diffusant interaction with this medium, *ie.* the diffusion coefficient. The adsorption and desorption processes are affected by the relative affinity of the permeant for the donor/receptor phase and the membrane, *ie.* the partition coefficient. From the data in Table XIX it can be seen that the permeability coefficient for each membrane increases, almost linearly, with experimental temperature. The lag times to steady-state permeation are also in close, inverse, agreement with the temperatures for most of the membranes. These quoted lag times should be regarded as rough

through this silicone media.

These temperature permeation data may also be applied to the calculation of the activation energies that, theoretically, estimate the ease with which the molecule diffuses through the membrane. Generally, the events governing the diffusion process may be more apparent by examination of the activation energies for each membrane. In solution, where the solute molecules are relatively large in comparison to those of the mobile solvent, diffusion is relatively facile and activation energies are usually in the range of 3-5 kcal mol^{-1}. In contrast, molecules diffusing through membranes may be relatively small compared to the polymer or molecular aggregate structures with which they interact. These membrane structures are also reasonably fixed in their spatial arrangement, thereby further adding to the diffusive resistance. Activation energies in these circumstances are concomitantly higher.

Estimation of the activation energy may, thus, give some indication of the extent of interaction of the diffusant with the molecular structures of the diffusion medium. Low activation energies would suggest diffusion is occurring through isopropyl myristate-filled channels. Membrane/fluid partition coefficients close to unity would support this theory by implying that the molecules are partitioning from the donor solution into the solvent-filled pores rather than being solubilized by the molecular structures of the membrane. In contrast, high activation energies may suggest extensive interaction of the diffusant with these structures by solubilization or molecular bonding. Activation energies may be derived from the Arrhenius principle:

$$\log D = \log D_o - E / R T \qquad \text{Equation 19}$$

where D is the diffusion coefficient, D_o is the diffusivity at infinite temperature, E is the energy of activation, R is the universal gas constant and T is the temperature. Plots of log D *versus* reciprocal temperature should yield straight lines of gradient E / 2.303 R, from which the activation energies may be calculated. Figure 29 is a plot of this nature for the human and weanling pig stratum corneum data which demonstrates the typically linear Arrhenius relationship between the temperature and the diffusion parameter. The hairless mouse skin and silicone membrane data produced similar, linear graphs, of different dimensions, although the latter media exhibits a biphasic plot for temperatures below 25 °C and above 30 °C. The activation energies for the different membranes calculated from the gradients of these plots are listed in Table XX.

The silicone membrane exhibits fairly low activation energies that

estimates since they are sensitive to the placement of the regression line through the data. However, the expectation that the lag times would be shorter at higher temperatures is supported by the data for each medium. The silicone membrane exhibited an unexplained, biphasic pattern, having disproportionately shorter lag times at 30 °C and above, compared to lower experimental temperatures. The calculated diffusion and partition coefficients for this membrane exhibit a similar biphasic pattern, relatively higher diffusion and lower partition coefficients at higher temperatures.

The increases in calculated diffusion coefficient values with temperature are in good agreement for the human, weanling and mouse media. Mention should be made of the magnitude of the diffusion coefficients for the hairless mouse skin that are approximately 1000-fold greater than values for the human and weanling pig media. It is assumed this difference is an artefact of the calculation which uses the thickness of the whole mouse skin (0.07 cm) to compute the diffusion coefficient. While full thickness skin was used in the experimentation, it should be borne in mind that the stratum corneum (5.8 μm thick) is the major barrier to diffusion, and underlying strata further contribute relatively little to the resistance of the membrane. The effective thickness of the hairless mouse membrane, of uniform resistance, should therefore be considerably less than the 0.07cm value used in these calculations. An effective thickness of 0.0032 cm, for example, would reduce the resultant diffusion coefficients to values more in keeping with those of the human and pig tissues. The permeation data for these theoretical calculations, using an effective membrane thickness value, are also included in Table XIX for comparison.

The partition coefficients of the corticosteroid between isopropyl myristate and membrane are essentially independent of temperature for the animal membranes. The weanling media exhibits deviation from this generalization at 40 °C, again assumed to be a result of the nonideal preparative techniques that may have impaired the barrier potential of these media and, thereby, affected these calculations. Noteworthy here is the agreement of the partition coefficient data with the drug disposition data presented above in Table XVIII. The human and weanling horny layers have relatively large partition coefficients, implying affinity of the corticosteroid for these strata, and these media also exhibit the largest relative percentage reservoir of the permeating drug.

In contrast, the silicone membrane repeats its biphasic character in the magnitudes of the partition coefficients at low and higher temperatures. These data would suggest there may be definite temperature related factors governing the partitioning or diffusion of betamethasone 17-valerate into or

TABLE XX: Activation energies calculated from Arrhenius plots.

Membrane	Activation energy (kcal mol^{-1})
Silicone (<25 °C)	4.5333
Silicone (>30 °C)	2.7335
Human	19.3193
Weanling pig	14.4722
Hairless mouse	28.6619

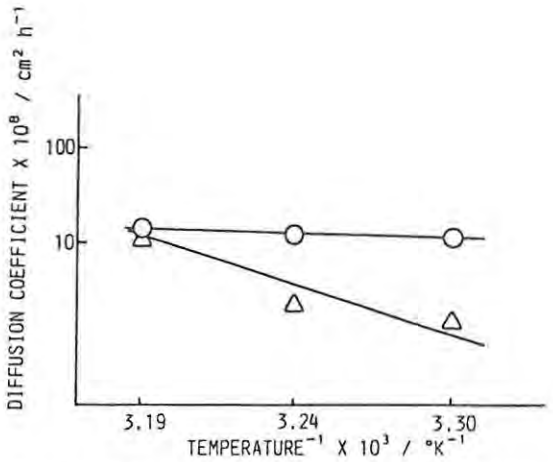


FIGURE 29: Arrhenius plot of diffusion coefficients for human (△) and weanling pig (○) stratum corneum.

would suggest betamethasone 17-valerate diffusion through this medium is a relatively facile process, possibly occurring through the isopropyl myristate-filled spaces between the silicone polymer chains. These values contrast with the 12.5-15.7 kcal mol^{-1} range reported by Ghannam *et al. (120)* and Lee *et al. (124)* for the permeation of progesterone derivatives through silicone medium. This difference is, again, ascribed to the greater degree of lipophilicity of the betamethasone moiety. On the other hand, diverse solubilization and bonding interactions are expected in the heterogeneous environment of the animal membranes that would increase the difficulty of the diffusion process. This assumption is supported by the activation energies for the animal membranes that are extensively higher than that for the synthetic medium.

The activation energy calculated for the hairless mouse skin is higher than the 19 kcal mol^{-1} value reported by Durrheim *et al. (107)*. This is, again, presumed to be an artefact of the skin thickness used in the experiments. The use of whole mouse skin causes the estimated lag time to be approximately half the value obtained for weanling or human tissue at each temperature. However, the permeability coefficient for the mouse skin, calculated from steady-state data, is generally 5 to 11 times greater than values from the other animal media, and not twice as great as would be expected from the lag times. There is, therefore, an artificially long equilibration period for the mouse skin prior to the onset of steady-state permeation. This prolonged lag time, along with the membrane thickness, are used in the calculation of the diffusion and partition coefficients and both may contribute to the inaccuracies of the permeation parameter and activation energy estimations.

In summary, the new diffusion cell system appears to be adequately sensitive to experimental temperature for use in drug permeation experiments. This sensitivity has been demonstrated with both synthetic and animal membranes of differing thicknesses and compositions. The provisos for steady-

state permeation conditions are not violated during the time-course of typical experimentation. The results from this study agree closely with reported observations regarding the temperature dependence of the permeation parameters such as lag time, permeability and diffusion coefficients. Furthermore, the reported invariance of partition coefficient with temperature for the animal and synthetic media has clearly been demonstrated by this system. Variance in the permeation data observed has been explained in terms of sampling technique for the weanling pig stratum corneum and membrane thickness for the hairless mouse skin. These results, when considered with the relatively rapid temperature equilibration time for the isopropyl myristate-filled chambers (approximately four minutes for the lipid phase to heat from room temperature to 35 °C), suggest that the cell design is of sufficiently high standard to maintain precise thermal control over the permeation process. Having established the sensitivity of the system, a temperature of 35 °C was chosen for all further permeation experiments as one close to physiological conditions. This temperature would also maintain a thermal gradient across the membrane and applied formulation, using the *in vivo*-mimic cell configuration, that approximates clinical usage of the product.

4.2 PERMEATION OF BETAMETHASONE 17-VALERATE THROUGH SYNTHETIC MEMBRANES

In vitro drug permeation research would be greatly simplified by the availability of a synthetic medium that adequately simulates absorption through human or animal skin. A man-made membrane could, theoretically, be totally uniform in its barrier properties and this would minimize intersample variation. While it is dubious that a synthetic medium could possibly approach the heterogeneous complexity of the dermal strata, it may be possible to synthesize a membrane with specific characteristics that simulate certain of the dermal absorption processes, and thus may be applicable in certain laboratory diffusion experiments.

Having validated the performance of the newly designed *in vitro* diffusion cell, an investigation was undertaken into the permeation of betamethasone 17-valerate through various synthetic media and egg-shell membrane. Cellulose membrane has been used extensively in diffusion cell experiments with variable success (see Section 2.2). Turakka *et al.* *(64,71)* have used a porous polycarbonate filter membrane, and Washitake *et al.* *(93)* have reported on the benefits of using keratinaceous egg-shell membrane as the barrier medium.

The intrinsic diffusivity of the corticosteroid was monitored through these membranes, along with a silicone medium, to assess the rate of permeation and the variance exhibited by each.

Bichamber, steady-state diffusion experiments were conducted (Section 3.5) using a 0.1% solution of betamethasone 17-valerate in isopropyl myristate as the donor phase and purified isopropyl myristate as the receptor fluid. All experiments were conducted at 35 °C and 600 rpm internal stirrer bar agitation. The membranes were prepared by techniques described in Section 3.3. The cellulose membrane was prepared by aqueous soaking at three different temperatures (20, 60 and 97 °C for 60 minutes) which, it was believed, would extract the plasticizers, antioxidants and preservatives to variable extents. The cumulative masses of corticosteroid permeating unit area of each membrane per unit time are listed in Table XXI for silicone, egg-shell membrane and Nuclepore filter medium, and Table XXII for cellulose media. These values are plotted graphically in Figure 30.

TABLE XXI: Mean drug mass permeating unit membrane area at 600 rpm agitation and 35 °C. ($\mu g\ cm^{-2}$).

Time (hours)	Membrane		
	Silicone	Egg-shell	Nuclepore filter
0.5	8.66 (0.76)	11.31 (26.63)	130.60 (98.95)
1.0	19.71 (0.08)	52.21 (42.16)	281.89 (114.64)
1.5	31.44 (0.08)	79.90 (56.63)	392.26 (145.18)
2.5	53.65 (0.67)	138.53 (97.58)	-
4.0	91.87 (0.65)	204.58 (151.18)	-
6.0	137.92 (0.37)	303.13 (232.81)	-
9.0	199.41 (4.78)	463.01 (347.26)	-
12.0	266.06 (0.71)	-	-
CC	0.9991	0.9971	0.9960

Standard deviations of means in parentheses.
CC Correlation coefficient.

TABLE XXII: Effect of pretreatment on drug mass permeating unit cellulose membrane area at 600 rpm agitation and 35 °C. ($\mu g\ cm^{-2}$).

Time (hours)	Membrane pretreatment (60 minutes)		
	Soaked 20 °C	Soaked 60 °C	Boiled
0.5	-	5.19 (0.55)	11.23 (1.01)
1.0	-	11.11 (1.49)	24.04 (2.87)
1.5	1.03 (0.11)	16.85 (1.90)	36.91 (3.69)
2.5	1.62 (0.69)	26.97 (3.25)	61.06 (5.81)
4.0	4.50 (2.82)	44.12 (5.37)	100.09 (7.91)
6.0	10.56 (3.76)	65.90 (5.88)	150.25 (8.01)
9.0	21.16 (4.14)	98.80 (6.08)	231.51 (17.98)
12.0	30.20 (7.11)	134.57 (8.26)	297.64 (9.42)
CC	0.9995	0.9998	o.0.9997

Standard deviations of means in parentheses.
CC Correlation coefficient.

Immediately apparent from this data is the large difference in the drug permeation rate through these synthetic media compared to the animal membranes reported in Section 4.1. Even hairless mouse skin, the most permeable of the animal samples studied, exhibited an average permeant mass of only 123 $\mu g\ cm^{-2}$ after 72 hours of experimentation. In this investigation 20 °C-soaked cellulose membrane, the least permeable of the synthetic media, exhibited a

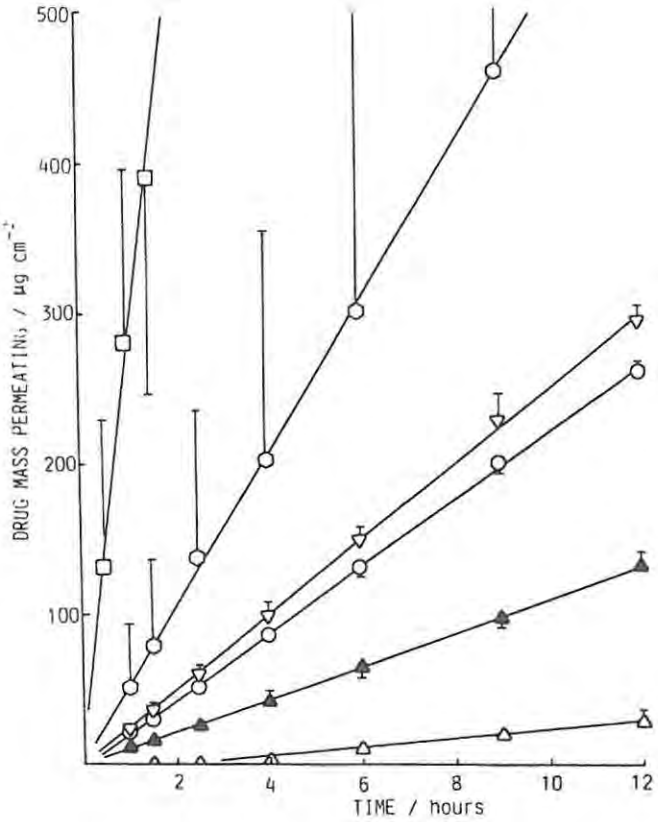


FIGURE 30: Permeation of betamethasone 17-valerate through various synthetic membranes at 600 rpm agitation and 35 °C. △ 20 °C-soaked cellulose, ▲ 60 °C-soaked cellulose, ▽ boiled cellulose, ○ egg-shell, □ porous filter, ○ silicone.

permeant mass of approximately 30 µg cm^{-2} after only 12 hours. If one compares the data for human stratum corneum with that from the egg-shell or filter membranes, this difference in barrier potential is even more marked.

Also noteworthy is the magnitude of the standard deviations for the egg-shell and filter membranes. Permeation experiments were conducted in sextuplicate for each of these membranes, double the number for the other media, because of the gross variance observed in the results. The filter membrane offers the least barrier to permeation, as expected from a porous medium, and has correspondingly high standard deviations at each sampling interval. It is anticipated that diffusion occurs simply through the fluid-filled pores that penetrate the membrane, resembling a sieving process, with very little interaction between diffusant and the polycarbonate medium. The pore diameter is very large in comparison to the steroid molecule size and, hence, no physical rate-limiting function is imposed on their passage. The diffusive resistance of the egg-shell membrane is probably the least precise of the media tested and, interestingly, the permeation rate correlates inversely with visual opacity of each specimen, thicker membranes presenting a greater barrier to corticosteroid movement. The samples exhibited the normal biological variability in their thickness and no correlation was apparent between the size of the egg and the thickness of the prepared membrane.

As anticipated, the pretreatment of the cellulose dialysis membrane markedly affects its barrier properties. Presumably, boiling the commercial product maximally removes plasticizers and other additives and causes the membrane to hydrate and swell appreciably. Simply soaking the membrane at 60 °C has lesser extractant effect on these additives. The 20 °C-soaking treatment produces a medium that is relatively impervious to the corticosteroid, compared to the other cellulose membranes, and most closely approaches the barrier potential of hairless mouse skin. Furthermore, in all pretreatments the cellulose media exhibited fair precision in its performance, as exemplified by the relatively small standard deviation bars in Figure 30. These foregoing observations imply that cellulose media may be useful in assessing drug permeation in the laboratory.

In this investigation silicone membrane produced the most precise, reproducible results from a large number of replicate experiments (standard deviations in most cases are too small to plot on the graph). This is ascribed to the totally aporous nature of the medium which dictates that diffusant molecules pass between the polymer chains, thereby inducing some degree of rate-limiting function to the permeation process.

Statistically, all the data points at the 0.5-hour interval are not significantly different from neighbouring points. Furthermore, the difference in the mean permeant masses for the boiled cellulose and silicone membranes are also not significantly different at the 1-hour interval. However, the large standard deviations for the egg-shell membrane make its mean values statistically equivalent to those of the cellulose (boiled and 60 °C-soaked) and silicone media at all sampling times. As previously discussed, this suggestion of statistical equivalence is debatable as the mean data values for the egg-shell medium are obviously greater than those of the other media.

An examination of the various permeation parameters may produce further useful insight into passage of drug through these membranes. The permeability coefficients are easily obtained from the flux values. Knowing the thickness of the membrane, the diffusion coefficient may be calculated for each medium, as before, by extrapolating the least squares, steady-state plots to the time axis and thereby estimating the lag period. Similarly, the partition coefficient of the permeant between isopropyl myristate and the membrane may be calculated. The estimations of these parameters are listed in Table XXIII and the equivalent values for human and animal media (from Section 4.1) are included for comparison. As previously stated, the determination of the lag time is open to significant error in the placement of the least squares regression line and, therefore, these calculated parameters can only be regarded as estimates. Notwithstanding this possible error, their rank

methasone by these authors. As with other membranes, the hydrophilic-lipophilic characteristics of the permeant are major determinants of the flux rate. This theory is exemplified by the results of Touitou and Abed *(108)* who report, in contrast to the above results, that cellulose membrane was three to four times more permeable to the smaller, hydrophilic benzoic acid moiety than either silicone or hairless mouse skin.

The lag time and partition coefficient for the 20 °C-soaked cellulose are large relative to the membranes pretreated at higher temperatures. The longer lag time suggests a greater degree of binding within the medium. As all the experiments were conducted at 35 °C, there would be no thermal-induced difference in the degree, or transience, of binding to the available sites; any observed increase in this interaction must, therefore, be generated by an increase in the total number of binding sites available. It is suggested that these additional sites are provided by the cellulose additives (plasticizers, preservatives and antioxidants) that have not been extracted from the medium by the cool water soaking process. The relatively large, calculated partition coefficient supports this hypothesis in that it suggests there is a fairly large affinity of the membrane for the diffusant molecules, an affinity that is not paralleled in cellulose membranes where additive extraction is more extensive. Similarly, the interaction of the corticosteroid molecules with these additives would slow the permeation rate through the medium, as exhibited by the flux profile.

In summary, it may be concluded that porous filter and egg-shell membranes are of limited use in diffusion cell experiments. The filter appears to offer negligible rate-limiting barrier to the permeant passage and it is suggested this medium may be useful only for dosage form containment purposes where no barrier potential is required, for example in the ointment jar experiments where topical products are immersed in the receptor medium. Similarly, the variability in thickness of the egg-shell membranes, and their diffusive resistances, produces imprecise results that are unsuitable to scientific investigation of this nature. On the other hand, the uniformity experienced with cellulose dialysis medium indicates that this may be a useful synthetic model. The 20 °C-soaked cellulose appears especially promising in that, of the media studied, the magnitude of the drug flux passing through this barrier approximates that of the animal membranes most closely.

Alternatively, the precision observed with the silicone membrane is unparalleled by the cellulose media and this factor may prove more beneficial in permeation data acquisition. This would be especially important where the donor vehicles may differ very slightly from one another and precise results

ordering should be accurate and this alone should be sufficient to draw certain conclusions concerning the permeation process.

TABLE XXIII: Calculated permeation parameters for betamethasone 17-valerate across synthetic and biological membranes at 35 °C and 600 rpm agitation.

Membrane	Thickness (cm)	Lag Time (hours)	Permeability coefficient (cm h^{-1} x 10^4)	Diffusion coefficient (cm^2 h^{-1} x 10^6)	Partition coefficient
Filter	0.01	-0.0270	2684.37	60000 *	0.0045 *
Egg-shell	0.0082	0.0351	480.894	319.278	1.2351
Cellulose boiled	0.009	0.0365	245.136	369.863	0.5965
Silicone	0.0127	0.0581	221.181	462.918	0.6068
Cellulose 60 °C	0.009	0.0418	109.648	322.967	0.3056
Cellulose 20 °C	0.009	2.6402	15.1486	5.1133	2.6664
Hairless mouse	0.07	3.1685	14.8823	257.748	0.4042
Weanling pig	0.0026	6.1309	2.7428	0.1895	3.8218
Human	0.0017	8.6525	1.2727	0.0544	3.9329

* Using lag time estimation of 1 second.

The magnitudes of the permeation parameters for silicone and the animal membranes have been discussed in Section 4.1. The estimated lag time for the filter membrane yields a negative value and, thus, an equilibration time of one second, believed to be realistic in the diffusion situation, has been used in the calculation of the diffusion and partition coefficients. This negative estimation would suggest that there is essentially no distribution of the diffusant within the medium and permeation ensues immediately upon contact of the donor solution with the filter. The diffusion coefficient thus calculated is extremely large, supported by the gradient of the plot in Figure 30, and the resulting partition coefficient is, therefore, fairly small. The partition coefficient for the egg-shell membrane is slightly greater than unity which may suggest a significant degree of adsorptive or solubilization interaction between diffusant and the keratin environment of the medium.

The estimated lag times for the cellulose membranes (boiled and 60 °C-soaked) are approximately twice that reported by Barry *et al. (13,97)* for dexamethasone permeating cellulose membrane. The permeation parameters for dexamethasone and betamethasone are expected to be fairly similar, the two molecules differing only in the configuration of the methyl substituent at the C-16 position. In this case it is postulated that the lipophilic valeroxy substituent on the betamethasone will decrease the tendency of the molecule to adsorb the relatively hydrophilic groups of the cellulose polymers. This will increase the transience of bonding within the membrane and will, thereby, reduce the lag time in relation to the more hydrophilic dexamethasone. These factors would also account for the greater permeability coefficient observed for the betamethasone 17-valerate in comparison to that reported for dexa-

would, therefore, be required to distinguish different drug release rates. The latter two membranes were, therefore, selected for further investigation using a commercial cream dosage form as the donor vehicle.

4.3 PERMEATION OF BETAMETHASONE 17-VALERATE FROM TOPICAL DOSAGE FORMS

The ultimate test of the *in vitro* diffusion cell system would be the assessment of its ability to distinguish between the drug release rates from very similar formulations and, furthermore, correlate these differences with those observed *in vivo*. Having validated the functioning of the cell design, and having conducted a preliminary investigation into possible synthetic media for use in the system, the main body of the research concerning the betamethasone 17-valerate release and permeation from commercial topical dosage forms was initiated. The *in vivo*-mimic technique, as described in Section 3.5, was adopted for all these investigations in which a single receptor chamber was used and an infinite dose of formulation (cream, lotion, ointment or scalp application) was applied to the donor surface of the membrane under investigation. Permeant concentration was monitored in the receptor isopropyl myristate solution by high-performance liquid chromatography as described previously. Full details of the commercial products tested are presented in Appendix 1 along with their assay values. All experiments were conducted at 35 °C and 600 rpm stirrer bar agitation.

COMMERCIAL CREAMS

The proprietary, 0.1% betamethasone (as the 17-valerate) creams tested in this investigation were Betnovate, Celestoderm-V and Persivate. Permeation was measured through cellulose soaked in water at 20 °C, silicone membrane, human and weanling pig stratum corneum, and full thickness hairless mouse skin.

Initially the drug permeation from Betnovate cream was monitored through silicone and cellulose membranes so that selection of the most suitable synthetic medium, from this pair, could be made for use in further experimentation. The results of this comparison are presented in Table XXIV and plotted in Figure 31. The magnitude of the corticosteroid flux through these two media is of the same order, with less drug permeating the cellulose than the silicone membrane, and is in agreement with the magnitude reported by

TABLE XXIV: Mean drug mass permeating synthetic membranes from Betnovate cream at 35 °C and 600 rpm. ($\mu g\ cm^{-2}$).

Time (hours)	Membrane	
	Cellulose	Silicone
3	1.859 (0.064)	11.402 (5.057)
6	3.837 (0.841)	14.832 (5.536)
12	7.058 (1.861)	21.276 (6.379)
24	12.811 (4.975)	30.108 (7.177)
30	15.930 (7.775)	33.230 (5.093)
48	23.858 (11.479)	43.984 (5.796)
54	25.061 (11.944)	45.240 (4.963)
72	32.852 (16.800)	54.985 (6.364)

Standard deviations of means in parentheses.

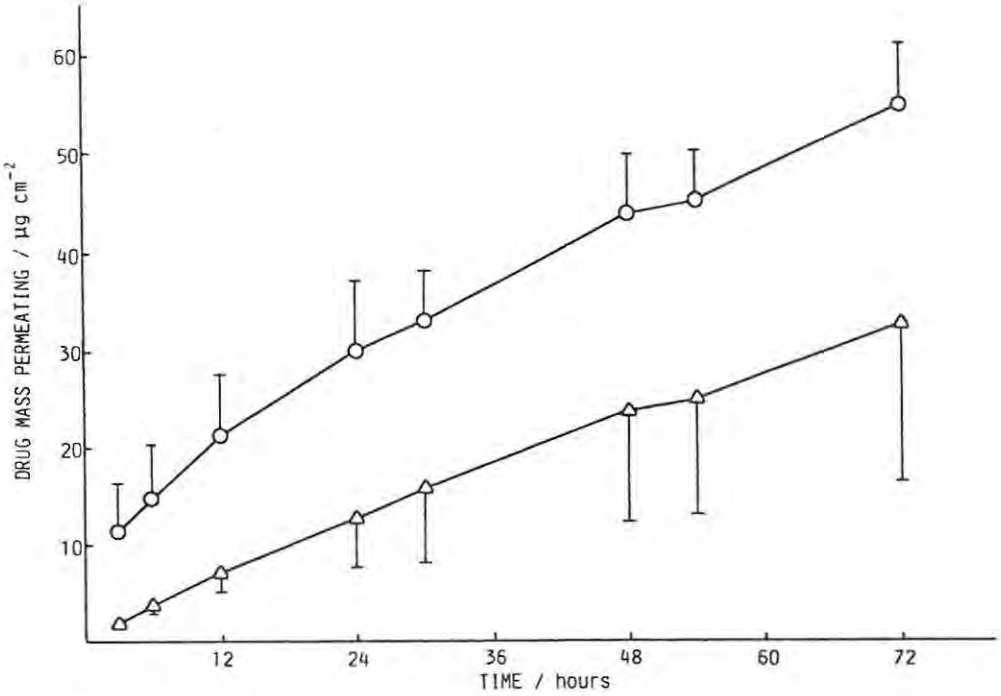


FIGURE 31: Permeation of betamethasone 17-valerate through cellulose and silicone membranes from Betnovate cream at 600 rpm agitation and 35 °C. Δ 20 °C-soaked cellulose, O silicone.

Washitake *et al. (93)* for betamethasone 17-valerate permeation through cellulose membrane from a hydrophilic ointment vehicle. Statistical equivalence of the data values is only exhibited at the 72-hour sampling time, where the standard deviations of the means are relatively large for both membranes. However, after the 24-hour sampling time, the variance exhibited by the cellulose is greater than that observed for the silicone medium. The standard deviations quoted as a percentage of the actual permeant mass average 35.79% for the cellulose and 22.97% for the silicone membrane. This variance is especially apparent at the end of the experiment where the standard deviation for the cellulose attains 51% of the permeant mass while the equivalent value for the silicone membrane is only 11%.

It is proposed that the variance observed with the 20 °C-soaked cellulose medium is due to differential interaction of the membrane additives with the diverse constituents of the cream dosage form. The hydrophilic and

lipophilic constituents of the formulation may solubilize or adsorb the plasticizers, antioxidants or preservatives that have not been removed from the medium by soaking in cool water. Any solubilization or extraction of this nature would render the additives unavailable for interaction with the permeant molecules and the diffusive passage of the latter would, thus, be more facile. The extent of this solubilization interaction between additives and excipients must vary considerably from cell to cell to produce variance in permeation of the magnitude reported here. If the cream dosage form, as a whole, presents a favourable solvent for the hydrophilic membrane additives, then the variance expected with a lotion formulation should be even greater than that observed here. The less viscous lotions usually contain a large proportion of cosolubilizers and humectants, such as propylene glycol, that improve the solubilization potential of these dosage forms several-fold.

Conversely, the silicone membrane appears to perform relatively uniformly and similar solubilization problems are not expected with the silicone as there are no additives present in this medium. Therefore, on the basis of the large variance exhibited by the cellulose medium and the relatively better precision demonstrated by the silicone, it was decided to abandon further experimentation using the cellulose. It was believed that the silicone medium would be better able to distinguish between the drug release rates from commercial formulations of, presumably, similar composition.

Having selected the best synthetic membrane, *in vivo*-mimic permeation experiments were conducted to compare betamethasone 17-valerate release from three commercial cream preparations through the silicone medium, human and weanling pig stratum corneum, and full thickness hairless mouse skin. The data from these experiments are presented in Tables XXV-XXVIII and plotted in Figures 32-35, respectively. The drug flux rate exhibited through each membrane is lower than that observed in Section 4.2 where a 0.1% solution of betamethasone 17-valerate in isopropyl myristate was used as the donor vehicle. This would imply that release or partitioning factors between the cream formulation and membrane are more significant here in governing drug permeation rates than the intrinsic diffusivity of the drug through the membrane.

TABLE XXV: Mean drug mass permeating silicone membranes from cream formulations at 35 °C and 600 rpm. ($\mu g\ cm^{-2}$).

Time (hours)	Commercial preparation		
	Betnovate	Celestoderm-V	Persivate
3	11.402 (5.057)	9.476 (7.080)	16.006 (7.689)
6	14.832 (5.536)	13.873 (7.303)	19.851 (7.951)
12	21.276 (6.379)	21.008 (8.267)	28.152 (9.272)
24	30.108 (7.177)	28.282 (6.827)	37.076 (9.689)
30	33.230 (5.093)	32.794 (7.608)	41.465 (10.354)
48	43.984 (5.796)	41.650 (6.069)	51.259 (9.748)
54	45.240 (4.963)	45.027 (6.902)	55.689 (9.597)
72	54.985 (6.364)	50.924 (6.860)	63.727 (9.900)

Standard deviations of means in parentheses.

TABLE XXVI: Mean drug mass permeating human stratum corneum from cream formulations at 35 °C and 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation		
	Betnovate	Celestoderm-V	Persivate
24	1.379 (0.478)	1.096 (0.122)	1.695 (0.734)
48	2.294 (0.253)	2.585 (0.928)	3.773 (1.639)
72	3.438 (0.668)	3.755 (0.764)	5.625 (2.008)
96	4.366 (0.276)	5.143 (0.853)	7.207 (2.654)
120	5.104 (0.168)	6.212 (0.604)	8.303 (2.739)
144	6.691 (0.576)	7.111 (0.645)	9.898 (4.041)
168	7.285 (0.631)	8.378 (0.735)	12.262 (3.618)
192	8.125 (0.993)	9.407 (0.207)	13.418 (4.025)

Standard deviations of means in parentheses.

TABLE XXVII: Mean drug mass permeating weanling pig stratum corneum from cream formulations at 35 °C and 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation		
	Betnovate	Celestoderm-V	Persivate
24	1.077 (0.146)	1.204 (0.241)	1.820 (0.508)
48	2.286 (0.279)	3.189 (0.568)	3.943 (2.635)
72	2.940 (0.448)	4.688 (0.974)	5.576 (2.899)
96	3.737 (0.675)	5.530 (0.499)	7.273 (4.513)
120	5.161 (0.747)	6.773 (0.696)	8.951 (5.725)
144	5.930 (1.001)	8.471 (1.711)	11.022 (6.840)
168	6.467 (1.588)	10.115 (3.264)	12.420 (6.339)
192	7.865 (0.738)	11.455 (3.802)	13.446 (7.266)

Standard deviations of means in parentheses.

TABLE XXVIII: Mean drug mass permeating hairless mouse skin from cream formulations at 35 °C and 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation		
	Betnovate	Celestoderm-V	Persivate
16	3.727 (0.343)	3.341 (0.317)	2.968 (0.857)
24	4.736 (0.369)	4.874 (0.326)	4.157 (0.181)
48	8.780 (0.078)	7.778 (0.415)	7.760 (0.149)
72	12.059 (0.487)	14.005 (0.719)	11.490 (1.044)
96	15.906 (0.346)	17.631 (1.540)	15.435 (1.294)
120	18.892 (0.859)	19.405 (1.143)	18.343 (1.870)
144	21.860 (0.490)	22.902 (2.598)	20.539 (1.370)
168	24.799 (1.723)	26.728 (3.570)	25.583 (3.120)
192	28.477 (1.162)	29.976 (3.262)	27.475 (1.421)

Standard deviations of means in parentheses.

The rank order of drug permeation from the cream dosage forms through the silicone membrane is Persivate greater than Betnovate, and both of these are greater than Celestoderm-V, however there is no statistical difference between the three mean values at any of the sampling times. Corticosteroid release in this case appears to follow the square-root-of-time relationship, suggested by the characteristic nonlinear permeation profile. This is confirmed by conducting least-squares analysis on the data *versus* the square-root-of-time that yields correlation coefficients of 0.9983, 0.9993 and 0.9992 for the Betnovate, Celestoderm-V and Persivate formulations, respectively.

The magnitude of permeation through this polymer is approximately five-fold greater than that through human or pig membrane, and approximately twice that through the hairless mouse skin. These observations suggest that the rate-limiting step to permeation is not the intrinsic resistance of the silicone membrane, but the release rate of the drug from the dosage forms.

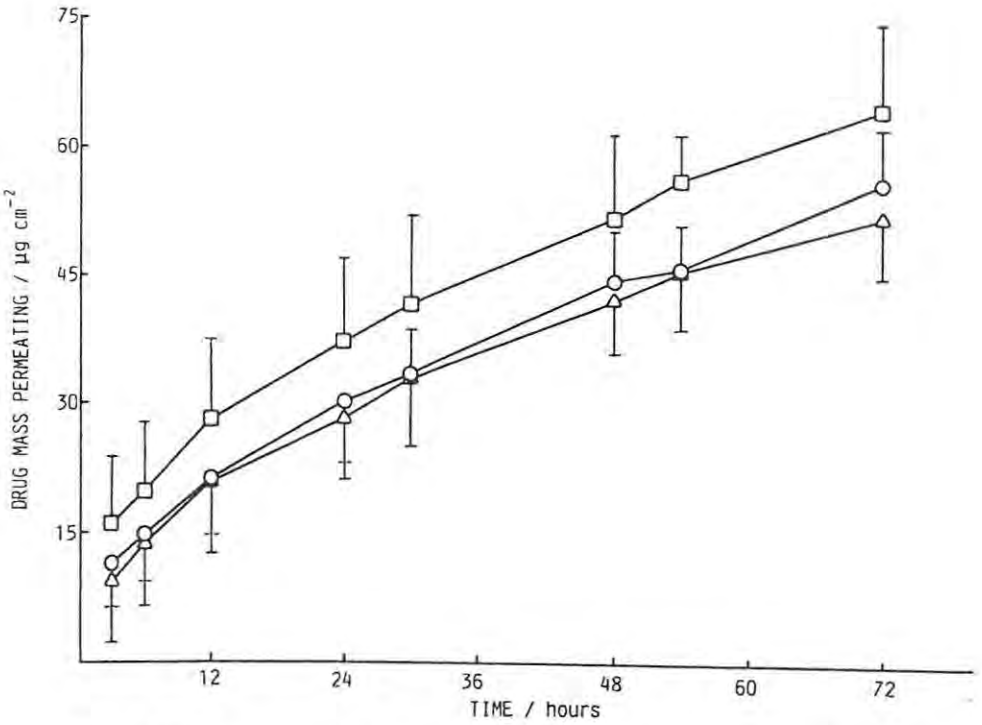


FIGURE 32: Permeation of betamethasone 17-valerate through silicone membrane from commercial creams at 600 rpm agitation and 35 °C.
○ Betnovate, △ Celestoderm-V, □ Persivate.

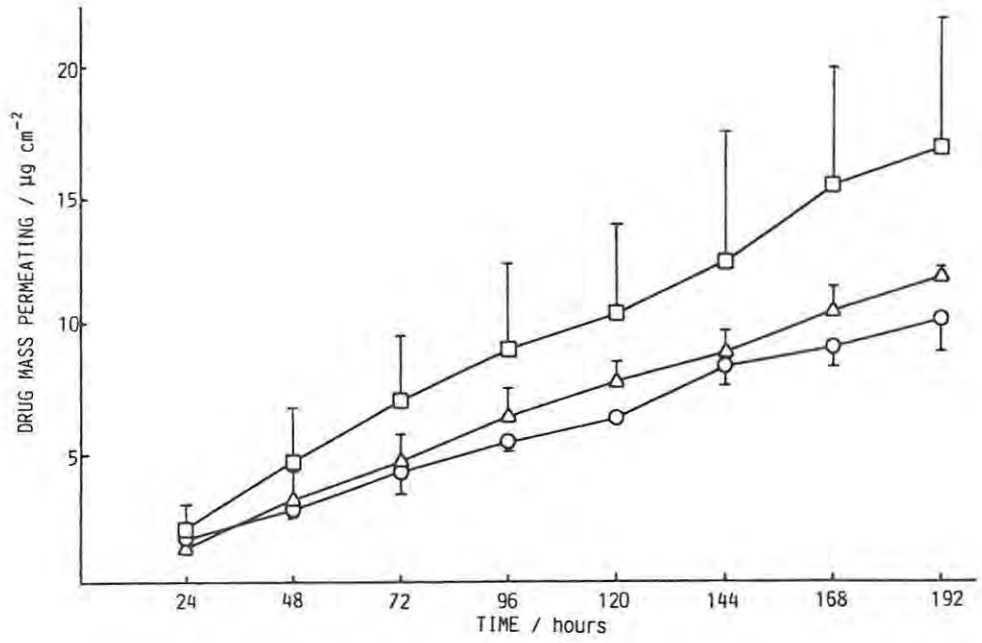


FIGURE 33: Permeation of betamethasone 17-valerate through human stratum corneum from commercial creams at 600 rpm agitation and 35 °C.
○ Betnovate, △ Celestoderm-V, □ Persivate.

Conversely, all three animal media present relatively linear permeation profiles over a longer experimental period, implying that the partitioning of drug between the cream and membrane may be the most dominant factor in the mass transfer process where these biological media are concerned. It must be

remembered that drug flux rates from the cream donor vehicles are slower than the membranes have been shown capable of supporting (Section 4.2). The intrinsic resistivities of the membranes are, therefore, not the rate-limiting functions to permeant passage here.

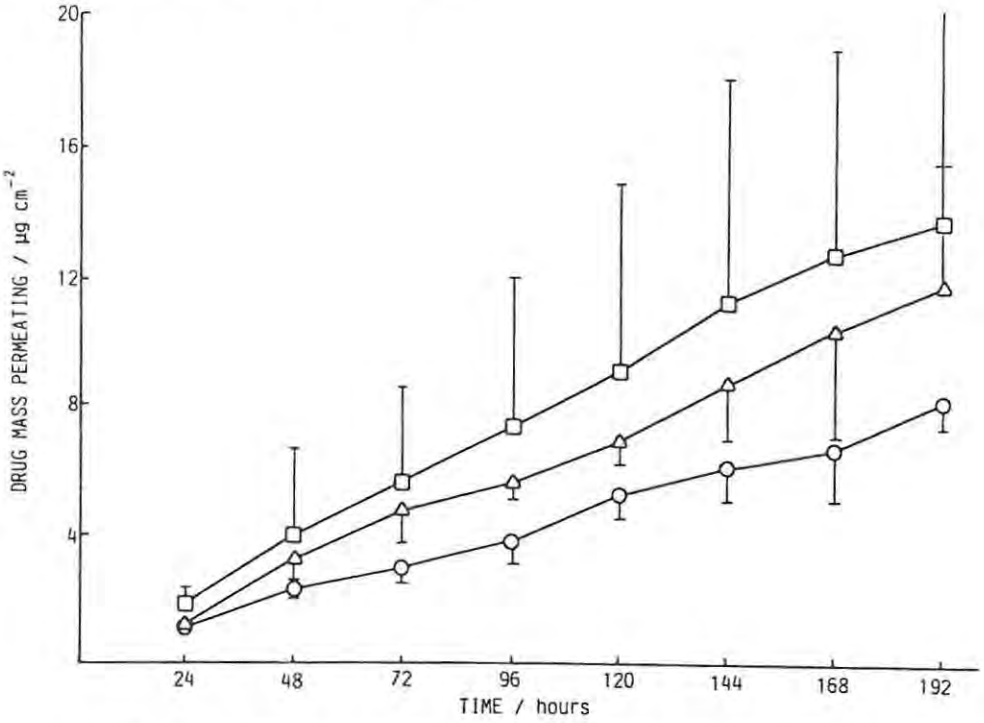


FIGURE 34: Permeation of betamethasone 17-valerate through weanling pig stratum corneum from commercial creams at 600 rpm agitation and 35 °C. ○ Betnovate, △ Celestoderm-V, □ Persivate.

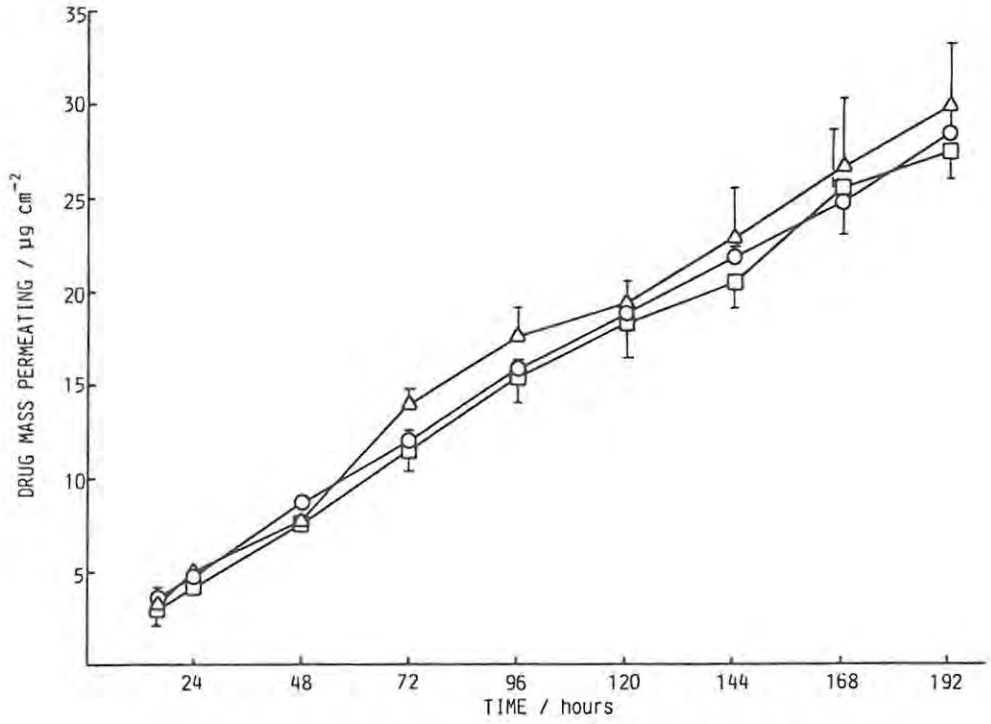


FIGURE 35: Permeation of betamethasone 17-valerate through full thickness hairless mouse skin from commercial creams at 600 rpm agitation and 35 °C. ○ Betnovate, △ Celestoderm-V, □ Persivate.

The rank order of drug permeation through the human stratum corneum from the commercial cream formulations is Persivate greater than Celestoderm-V, and these are greater than Betnovate. The magnitude of the flux experienced for this medium is approximately equivalent to that of the weanling pig membrane, and both of these tissues exhibit approximately one-third the permeability of the full thickness hairless mouse skin. As with the results from Section 4.1 and 4.2, these observation are interesting in view of the different thicknesses of membrane used to monitor permeation. There is statistical equivalence between the mean data values of all three preparations at all sampling times except the 120- and 192-hour periods. At these times, only the Betnovate and Celestoderm-V data means are significantly different.

The drug permeation rank order for the weanling pig stratum corneum from the cream donor vehicles is, in decreasing order, Persivate, Celestoderm-V and Betnovate (similar to the human tissue). However, with the porcine medium, the mean values for the Betnovate formulation exhibit more instances of statistically significant difference to those of the Celestoderm-V product when compared to the human tissue results. The Betnovate and Celestoderm-V data values are not statistically different at the 24-, 168- and 192-hour sampling times, whereas at all other periods there is significant statistical difference between the mean data values of these two formulations. Comparisons of the Persivate and Celestoderm-V, and Persivate and Betnovate means demonstrate statistical equivalence of the values at all sampling times except the 24-hour period.

The permeation rank order is more difficult to estimate for the hairless mouse skin, because of the proximity of the data points, but appears to be, in decreasing order, Celestoderm-V, Betnovate and Persivate. There is no statistical difference between the means of all three preparations at the 16- and 24-hour sampling times, and at all times between the 96- and 192-hour interval. The data means from the Persivate and Celestoderm-V creams are not statistically different at the 48-hour sampling time, as are the values for the Persivate and Betnovate formulations at the 72-hour period. Of the membranes studied, hairless mouse skin is unique in that the drug permeation rates from the different donor formulations are approximately equivalent, in comparison to the apparent differential rates observed for the other membranes.

The permeation results through the membranes studied using commercial cream dosage forms as the donor vehicles agree, in general, with observations reported in the literature, and with the results from the steady-state validation experiments reported in Section 4.1. Silicone membrane is more permeable

than full thickness hairless mouse skin, which is more permeable than either human or weanling pig stratum corneum, the latter two media exhibiting similar barrier potential. With all membranes, corticosteroid permeation is greatest from the Persivate cream donor formulation, except hairless mouse skin through which flux from this product is lowest. Permeation from the Celestoderm-V cream appears slightly superior to that from the Betnovate cream through all three animal media but not through silicone membrane.

Discrimination between the drug permeation rates from the different donor formulations is, therefore, possible using this *in vitro* diffusion system and either silicone or stratum corneum from human or porcine donors as the barrier medium. Rank order permeation is fairly consistent with all three of these media and is closest for the human and weanling pig membranes. The hairless mouse skin appears less able to discriminate between corticosteroid release from these topical cream formulations. This is possibly due to the thinner stratum corneum present in these skin samples that presents less of a resistive barrier to permeant passage. It is, therefore, proposed that the discriminatory ability of the animal membranes resides in the barrier potential of the stratum corneum. Membranes exhibiting relatively high flux rates are less able to distinguish the subtle release differences of similar formulations.

Notwithstanding these general observations, statistical evaluation of the data from all four membranes would suggest that there are relatively few instances of significant difference between the mean values. This evaluation would discount any differences in the observed drug permeation rates through the membranes as being, in most cases, artefactual. This is especially applicable when the Persivate dosage form is considered because of the relatively large standard deviations exhibited by the permeation data from this dosage form. However, a definite, general trend in the results is apparent. Bearing the normal membrane sample variability in mind, the mean permeation values suggest that some formulations are definitely superior to others in their drug release capabilities. In this case it is concluded that, on average, Persivate cream is superior to Celestoderm-V and Betnovate, respectively, in its ability to release betamethasone 17-valerate to the membranes studied.

EXTEMPORANEOUS CREAMS

The *in vivo*-mimic methodology was used to test the permeation of betamethasone 17-valerate from a number of extemporaneously prepared cream

formulations containing proportions of the possible penetration enhancing agents urea or resorcinol. The creams were prepared as described in Section 3.6 and consisted of a modified buffered cream base *(278)*, that had been formulated to have an apparent pH of 3.5, into which the corticosteroid was incorporated at a concentration of 0.12%. The cream formulations differed only in the concentration of penetration enhancer added. The various products tested contained 2% or 5% resorcinol, or 10% urea, and drug release from these products was compared to that from the formulation base containing neither resorcinol nor urea. Permeation was monitored at 35 °C from infinite doses of these formulations through full thickness hairless mouse skin, to a receptor phase of purified isopropyl myristate. Only one membrane type was used in this preliminary investigation as the objective was simply to assess the comparability of the results from the extemporaneous products with those from the proprietary cream formulations. The drug permeation data from these investigations is presented in Table XXIX and plotted in Figure 36.

TABLE XXIX: Mean drug mass permeating hairless mouse skin at 35 °C and 600 rpm from extemporaneously prepared buffered cream formulations containing 0.12% betamethasone 17-valerate and various proportions of either resorcinol or urea. ($\mu g\ cm^{-2}$).

Time (hours)	Extemporaneous formulation			
	No enhancer	2% resorcinol	5% resorcinol	10% urea
12	5.797 (0.947)	16.725 (5.069)	30.920 (0.750)	7.486 (0.166)
24	10.220 (1.404)	24.442 (6.116)	47.576 (1.517)	12.662 (1.805)
48	24.203 (2.106)	47.125 (7.203)	87.838 (4.410)	26.430 (1.758)
72	37.709 (2.077)	60.929 (6.185)	114.082 (4.142)	37.428 (1.799)
96	47.223 (2.497)	70.104 (5.138)	132.988 (15.077)	46.560 (2.653)
120	55.443 (2.430)	80.779 (5.311)	151.865 (10.987)	54.817 (1.205)
144	66.317 (2.276)	91.620 (4.271)	172.295 (13.782)	64.177 (1.410)
168	67.575 (2.599)	99.197 (5.164)	188.495 (12.896)	67.921 (2.683)
192	87.983 (2.699)	111.802 (4.833)	210.280 (19.349)	82.230 (2.806)

Standard deviations of means in parentheses.

It is immediately apparent that corticosteroid permeation from the extemporaneously prepared formulations markedly exceeds that from any of the commercial betamethasone 17-valerate creams. Even the buffered cream without enhancer and 10%-urea formulations, which exhibit the slowest drug release profiles of the extemporaneous formulations, produce a threefold greater drug permeation over the experimental period than Betnovate, Celestoderm-V or Persivate cream. Furthermore, the formulations containing 2% and 5% resorcinol produce approximately four- and sevenfold greater permeation magnitudes than the proprietary products. Statistically, only the mean data values from the cream without enhancer and 10%-urea formulations are equivalent at sampling times later than 24 hours. The differences between all other data values are statistically significant at all other sampling times.

Interestingly, with the extemporaneous formulations, the permeation profiles tend to be nonlinear, the flux rate decreasing with time. In contrast, drug permeation from the commercial creams through hairless mouse

skin (Figure 35) was essentially linear for all three products. For this reason it was proposed that the partition coefficient of the drug between the commercial formulations and skin was the rate-limiting function controlling the flux. This proposition was supported by the observation that the permeation rate had not nearly approached that shown possible by the steady-state experiments using an isopropyl myristate solution as the donor phase. On the other hand, drug permeation from the extemporaneous creams approaches, and exceeds, the magnitude of that observed in the steady-state experiments and, consequently, the profiles for certain of these formulations clearly demonstrate a square-root-of-time relationship. It is therefore suggested that the drug permeation rate from the formulations containing 2% and 5% resorcinol is governed by the intraformulation diffusion rate of the molecules attempting to replenish drug concentration at the membrane interface. As all other formulation factors are identical in all four extemporaneous products, the addition of resorcinol is obviously optimizing the partitioning of the drug into the mouse skin, as compared to the cream without resorcinol or the urea-containing product. The rate-limiting function in these cases has shifted from the partitioning mechanism to the speed of permeant replenishment on the donor surface of the membrane.

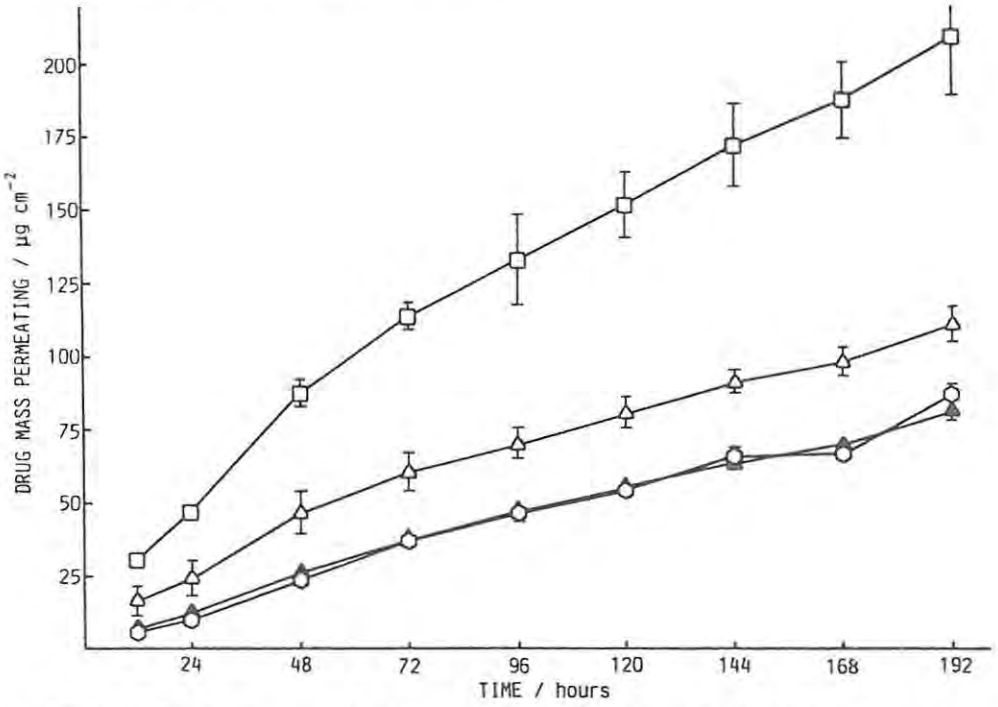


FIGURE 36: Permeation of betamethasone 17-valerate through full thickness hairless mouse skin from extemporaneously prepared buffered cream formulations containing resorcinol or urea at 600 rpm agitation and 35 °C. ○ 0% enhancer, △ 2% resorcinol, □ 5% resorcinol, ▲ 10% urea.

Linear regression analyses of the permeant masses *versus* the square-root of the time yield correlation coefficients of 0.9956 for the 5% resorcinol formulation and 0.9959 for the 2% resorcinol cream, indicating that, within

experimental error, these profiles follow an exponential decline in flux rate with time. The equivalent correlation coefficients for the cream without enhancer and the 10%-urea cream are 0.9884 and 0.9938, respectively, which indicate that the data in these cases do not fit a square-root-of-time profile that closely. It is probable, therefore, that these two profiles are intermediate between linear and exponential, and that permeation is governed, in similar proportions, by partitioning and the permeant replenishment rate at the membrane interface.

The mechanism by which resorcinol improves drug partitioning is unknown. The increase in flux rate is immediately apparent from the initiation of the experiment, which would discount a theory of barrier denaturation as, presumably, this would take several hours, or days, at this enhancer concentration. Furthermore, urea has been shown capable of denaturing the keratin matrix of the stratum corneum, and yet this is not evident from the permeation results.

It is also interesting to speculate on the optimization of drug release from the commercial formulations. One would assume that these products have been tested and modified to produce optimal drug release and stability, and yet a simple buffered cream formulation has been shown here to outperform these proprietaries, even without the resorcinol-enhancing effects. Furthermore, betamethasone 17-valerate stability in the buffered cream bases has been confirmed after two years of storage at room temperature, and hence these products are not unsuitable from a degradation point of view.

LOTIONS

The 0.1% betamethasone (as the 17-valerate) lotions tested by *in vivo*-mimic methodology in this investigation were Betnovate and Celestoderm-V, these being the only two commercial preparations on the South African market. Drug permeation was measured from an infinite donor dose of each formulation through silicone membrane, human and weanling pig stratum corneum, and full thickness hairless mouse skin to a receptor phase of purified isopropyl myristate. The data from these experiments are presented in Tables XXX-XXXIII, and plotted in Figures 37-40, respectively.

The corticosteroid mass permeating the silicone membrane clearly follows the square-root-of-time relationship, exemplified by the exponentially-shaped curves in Figure 37. This permeation pattern was suggested for the cream donor formulations, but is more obvious with the lotion vehicles, especially at the early sampling times.

TABLE XXX: Mean drug mass permeating silicone membrane from lotion formulations at 35 °C and 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation	
	Betnovate	Celestoderm-V
1.5	7.691 (0.554)	6.237 (0.279)
3	13.010 (0.619)	13.241 (2.153)
6	18.975 (0.511)	21.208 (1.362)
12	26.539 (0.505)	31.849 (1.179)
24	38.732 (4.150)	50.103 (0.944)
30	43.549 (2.854)	55.263 (2.558)
45	54.219 (6.933)	70.480 (0.606)
52	58.862 (5.731)	76.536 (2.559)
72	74.072 (3.338)	93.081 (1.098)

Standard deviations of means in parentheses.

TABLE XXXI: Mean drug mass permeating human stratum corneum from lotion formulations at 35 °C and 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation	
	Betnovate	Celestoderm-V
12	4.208 (1.950)	3.274 (2.075)
18	6.894 (2.218)	6.619 (5.088)
36	12.367 (2.437)	11.728 (4.139)
60	18.400 (3.121)	17.932 (4.905)
85	22.969 (2.695)	22.280 (5.885)
109	26.727 (3.248)	27.021 (6.128)
132	30.663 (4.099)	29.654 (7.285)
156	33.586 (3.798)	34.019 (8.196)
180	37.341 (3.805)	38.573 (8.828)
200	40.800 (4.675)	41.826 (9.292)

Standard deviations of means in parentheses.

TABLE XXXIII: Mean drug mass permeating hairless mouse skin from lotion formulations at 35 °C and 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation	
	Betnovate	Celestoderm-V
16	12.872 (1.024)	8.159 (2.111)
24	17.339 (0.466)	12.166 (3.599)
48	26.910 (3.337)	22.753 (3.134)
72	34.082 (5.247)	33.789 (2.114)
96	43.389 (7.244)	42.502 (2.213)
120	46.497 (9.040)	52.017 (1.277)
144	53.593 (9.802)	58.830 (1.027)
168	61.521 (4.442)	68.810 (5.009)
192	70.515 (1.392)	78.529 (7.445)

Standard deviations of means in parentheses.

TABLE XXXII: Mean drug mass permeating weanling pig stratum corneum from lotion formulations at 35 °C and 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation	
	Betnovate	Celestoderm-V
18	1.712 (0.836)	1.739 (0.512)
24	1.873 (0.663)	2.290 (0.778)
36	2.160 (0.414)	2.807 (0.866)
48	2.848 (0.709)	3.429 (0.805)
72	3.774 (0.319)	5.216 (2.039)
96	5.287 (0.484)	6.293 (2.965)
120	5.753 (0.900)	6.750 (2.698)
144	5.985 (0.956)	8.337 (3.346)
168	6.813 (1.105)	8.923 (3.160)
192	7.808 (2.111)	10.747 (5.181)

Standard deviations of means in parentheses.

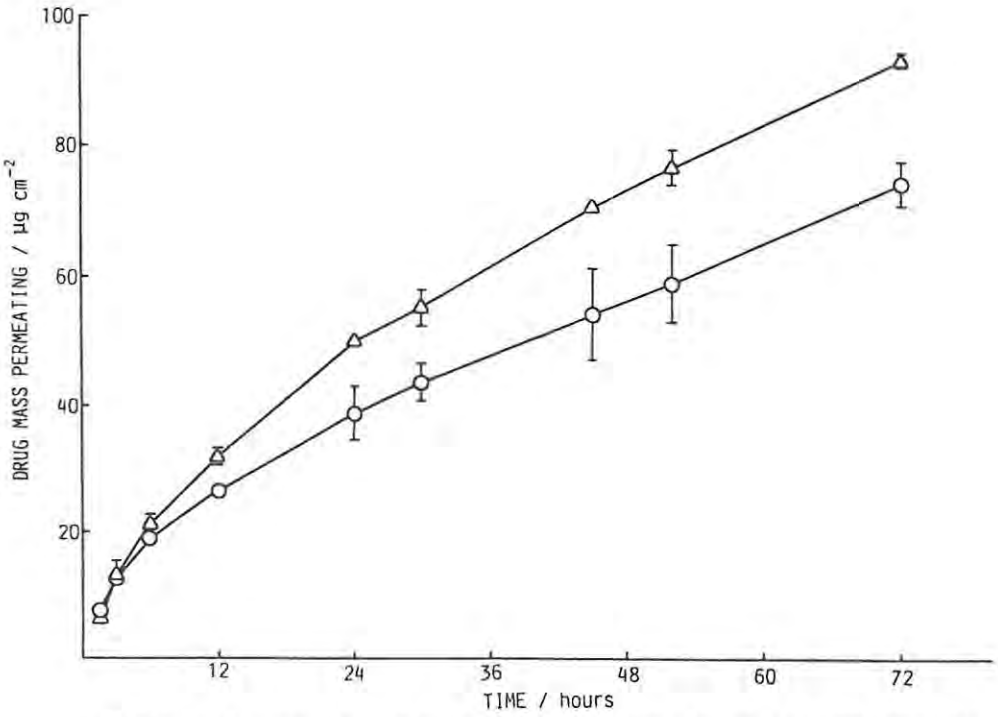


FIGURE 37: Permeation of betamethasone 17-valerate through silicone membrane from commercial lotions at 600 rpm agitation and 35 °C. ○ Betnovate, △ Celestoderm-V.

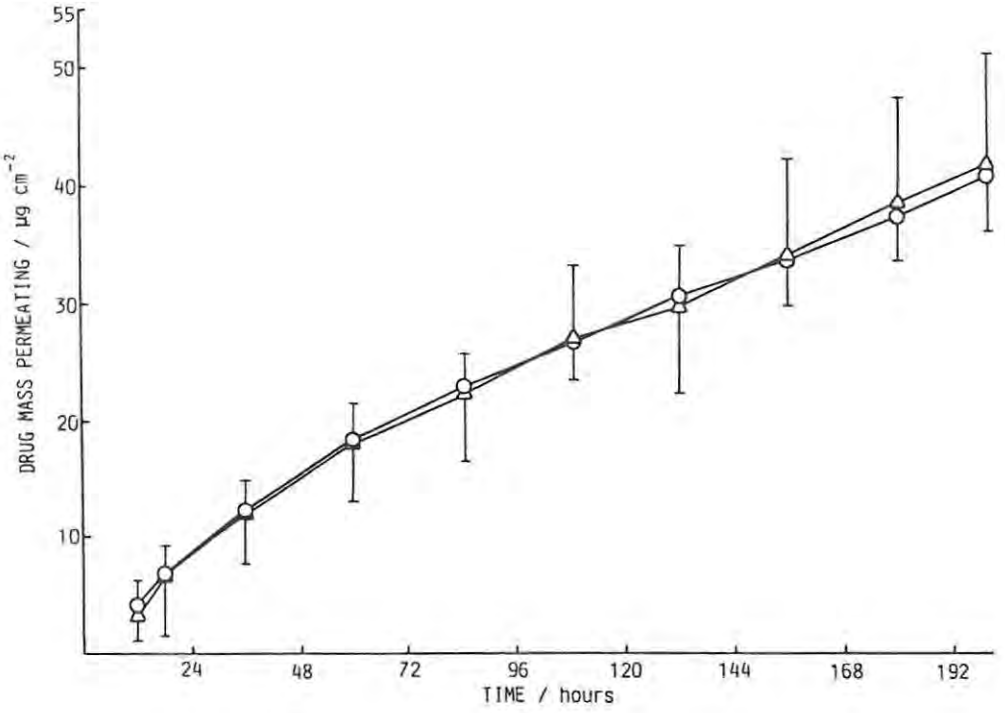


FIGURE 38: Permeation of betamethasone 17-valerate through human stratum corneum from commercial lotions at 600 rpm agitation and 35 °C.
○ Betnovate, △ Celestoderm-V.

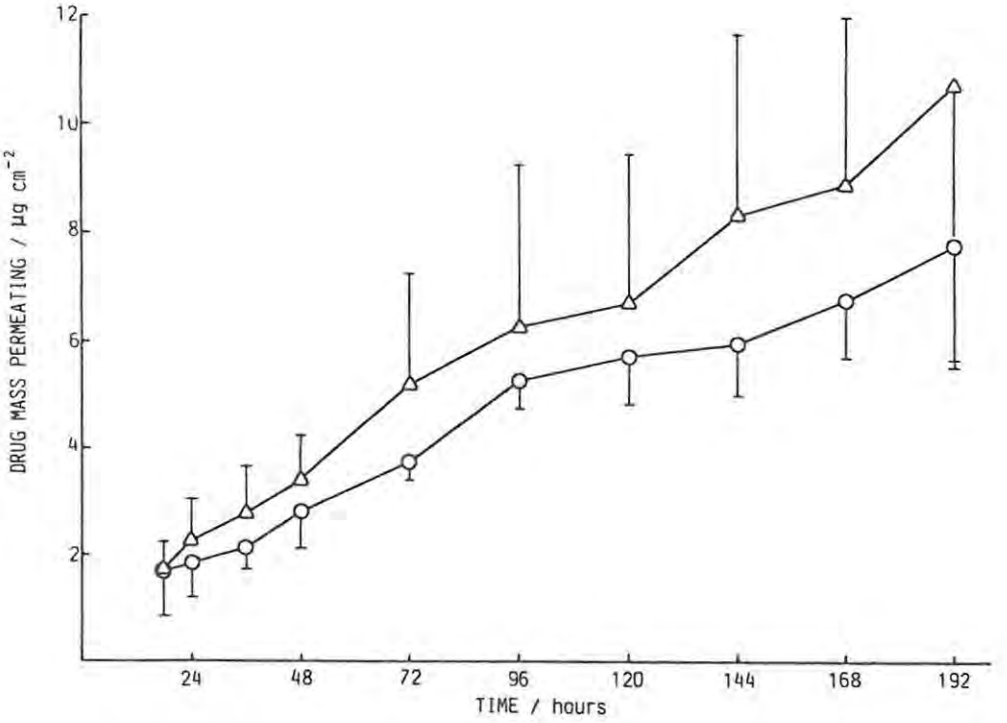


FIGURE 39: Permeation of betamethasone 17-valerate through weanling pig stratum corneum from commercial lotions at 600 rpm agitation and 35 °C.
○ Betnovate, △ Celestoderm-V.

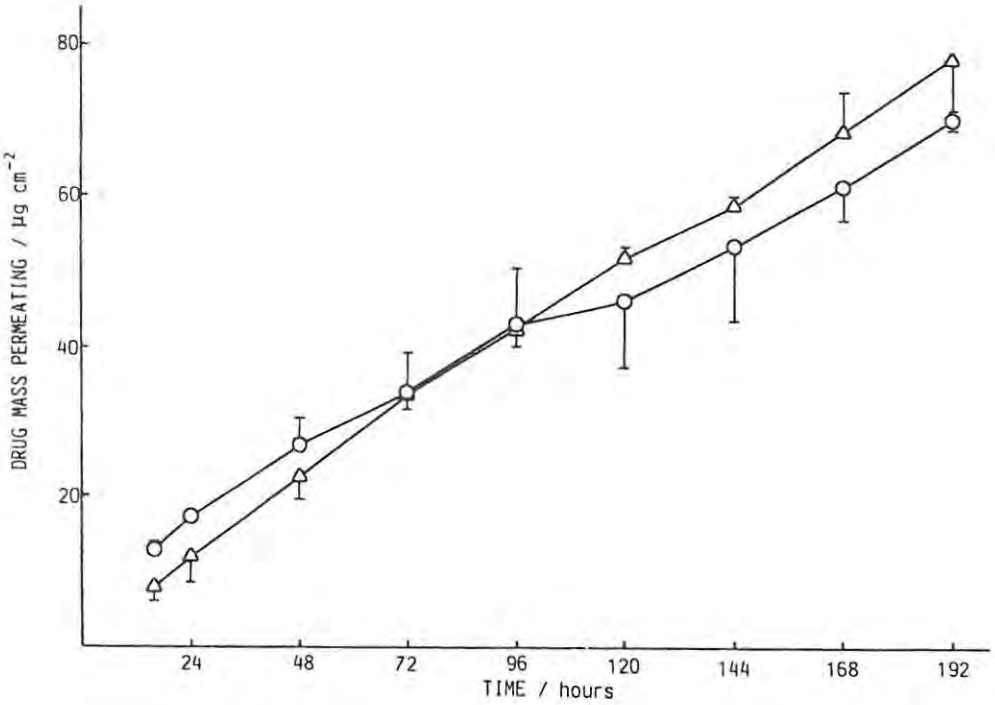


FIGURE 40: Permeation of betamethasone 17-valerate through full thickness hairless mouse skin from commercial lotions at 600 rpm agitation and 35 °C. O Betnovate, Δ Celestoderm-V.

Least-squares regression analyses of the permeant masses *versus* the square-root of the time values yields an ordinate intercept of -3.2, a gradient of 8.75 and a correlation coefficient of 0.9920 for the Betnovate formulation, and an intercept of -8.00, gradient of 11.77 and correlation coefficient of 0.9997 for the Celestoderm-V product. If it is assumed that the permeability capabilities of the silicone have not been saturated, then the replenishment of the permeant molecules at the membrane-lotion interface is the rate-limiting step to mass transfer in this instance. As molecules enter the membrane from the formulation they must be replaced at the interface by diffusion from the bulk donor phase if the flux rate is to be maintained. While this intravehicle diffusion may be fairly facile in the case of non-viscous solutions, it is more difficult in lotions and even moreso in the case of the viscous cream and ointment formulations and, hence, a decrease in flux rate is observed with time.

The Celestoderm-V formulation is superior to the Betnovate product in its ability to release betamethasone 17-valerate to the silicone membrane, although, at the 1.5-hour sampling time the average drug mass that has permeated from the Betnovate lotion is greater than that from the Celestoderm-V product. Statistically the mean flux values are significantly different at every sampling time except the intersecting, 3-hour period. The variance in the mean permeation data is greater for the Betnovate lotion than for the Celestoderm-V, as can be seen from the standard deviation bars. Furthermore,

the overall flux rate per unit time is greater from the lotions than from the cream dosage forms through this membrane. This is probably due to the more facile diffusion within the lotion environment, and possibly enhanced partitioning of drug between donor vehicle and membrane.

The corticosteroid permeation through human stratum corneum appears to follow a similar pattern to that through silicone, implying, again, that diffusion of the drug through the bulk lotion is the rate-limiting step. Least-squares regression analyses of the permeant masses *versus* the square-root of the time values yields an ordinate intercept of -7.59, a gradient of 3.34 and a correlation coefficient of 0.9993 for the Betnovate formulation; and an intercept of -9.08, gradient of 3.49 and correlation coefficient of 0.9978 for the Celestoderm-V product. The flux rate through human tissue from the lotions is markedly greater than that from the creams (an approximately fourfold difference), demonstrating the relative extent of drug partitioning in effect between the lotion or cream, and the stratum corneum. As both types of formulation present equivalent concentrations of betamethasone 17-valerate to the membrane, it is proposed that the drug partitioning from the creams is relatively lower than that from the lotions, hence the greater flux rates observed from the latter formulations.

Statistically, the two permeation profiles through human tissue are not significantly different, and this is clearly apparent from Figure 38. The drug permeation rate from Betnovate lotion appears to be slightly greater than that from Celestoderm-V at the earlier sampling times, with a reversal of this trend towards the end of the experiment, however, the plots are essentially equivalent.

The two plots representing corticosteroid permeation through weanling pig stratum corneum (Figure 39) are also statistically equivalent, mainly due to the large standard deviations of the mean data values. In this instance, permeation does not follow an obvious square-root-of-time pattern, and is greater from Celestoderm-V lotion than from the Betnovate formulation. In contrast to the human and silicone membranes, permeation through weanling tissue is not markedly greater from the lotions than from the cream donor vehicles. In view of the reasoning presented above, this may suggest that drug partitioning is similar between the stratum corneum and either formulation type, and, if this is the case, then the intrinsic resistivity of the membrane is governing the permeation rate.

Similarly, drug permeation through hairless mouse skin (Figure 40) follows a linear profile for both lotion formulations and intraformulation diffusion influences are not apparent from the graph. However, the extent

of drug permeation through the mouse skin from the lotions is approximately twice that from the cream dosage forms. As before, relatively better drug partitioning between lotion and skin, than between cream and skin, may account for these higher flux rates.

Statistical equivalence of the mean hairless mouse data values is exhibited at the 72-, 96-, 120- and 144-hour sampling times, where the permeation profiles of the two formulations intersect or are coincident. At all other sampling times the mean values are significantly different. In similarity to the human tissue data, drug permeation from the Betnovate formulation is greater than that from Celestoderm-V through the hairless mouse skin at the initial sampling periods, with an inversion of this order after the 96-hour period.

In summary, the data for the different membranes appear to follow one of two patterns. Drug permeation through the silicone membrane or weanling pig stratum corneum appears greater from the Celestoderm-V formulation than from the Betnovate product, although the two profiles for the porcine medium are not statistically different. On the other hand, human stratum corneum and full thickness hairless mouse skin produce very similar drug permeation profiles from both dosage forms, there being no statistical difference in the case of the human tissue, and certain instances of significant difference for the murine skin. An intersection of the profiles from the two lotions is also common to these two media, Betnovate lotion displaying apparent superior release characteristics at early sampling times while the average permeating mass of corticosteroid is greater from Celestoderm-V lotion at later sampling periods.

On the other hand, certain trends are common to both membrane groups. Both silicone and human membranes exhibit a decrease in flux rate with time. This is ascribed to the difficulty in molecular diffusion through the donor vehicle, in an attempt to replenish partitioned permeant at the interface. Both weanling pig and hairless mouse membranes exhibit linear permeation profiles, implying that partitioning or the intrinsic resistivity of the medium may be more influential in governing the extent of diffusion. Human and weanling pig membranes are alike in that they exhibit no significant statistical difference between the mean data values at any of the sampling times. Mouse skin does show significant superiority in the drug release from Celestoderm-V at certain periods, and silicone membrane displays this trend at all sampling times after three hours.

It has been established from the drug permeation data using the cream donor formulations that the animal membranes are capable of discerning small

differences in corticosteroid release rates. Considering only the data from the animal membranes, it must be concluded that the release profiles are equivalent for the two lotion formulations. Conversely, the silicone membrane data suggests that drug release is significantly greater from the Celestoderm-V than from the Betnovate lotion.

OINTMENTS

The commercial, 0.1% betamethasone (as the 17-valerate) ointments tested by *in vivo*-mimic methodology were Betnovate, Celestoderm-V and Persivate formulations. Drug permeation was measured from infinite donor doses of each formulation through silicone membrane, human and weanling pig stratum corneum, and full thickness hairless mouse skin, to purified isopropyl myristate. The data from these experiments are presented in Tables XXXIV-XXXVII, and plotted in Figures 41-44, respectively.

A general decrease in the betamethasone 17-valerate flux rate through silicone membrane is experienced with time for all three ointment donor formulations. These observations are similar to those reported in the cream and lotion experiments. As before, this decrease may be ascribed to the diffusive resistance experienced by molecules within the formulation attempting to re-equilibrate the permeant concentration at the membrane interface. Linear least-squares regression analysis of the mean permeant masses *versus* the square-root of the time for the silicone data yields an ordinate intercept of -42.97, a gradient of 34.14 and a correlation coefficient of 0.9967 for the Betnovate data; an intercept of -69.83, a gradient of 42.01 and correlation coefficient of 0.9896 for Celestoderm-V; and an intercept of -27.21, gradient of 25.08 and correlation coefficient of 0.9982 for the Persivate data. Therefore, within experimental error, these values support the hypothesis that drug permeation through silicone from ointments follows the square-root-of-time principle.

It is assumed that molecular diffusion within the bulk ointment vehicle would be relatively difficult because of the viscous consistency of these formulations. Nevertheless, drug permeation through the silicone membrane is approximately threefold greater than that from either the lotion or cream dosage forms. Bearing in mind the difficulties in diffusive replenishment of the permeant molecules at the interface, this flux magnitude must signify highly favourable conditions for corticosteroid partitioning from the lipophilic formulation to the polymer environment of the membrane. Mass transfer of this order can only be maintained if the affinity of the drug

for the membrane is much greater than its affinity for the donor vehicle.

TABLE XXXIV: Mean drug mass permeating silicone membrane from ointment formulations at 35 °C & 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation		
	Betnovate	Celestoderm-V	Persivate
1.5	10.180 (2.810)	6.431 (2.244)	8.950 (3.703)
3	17.088 (5.645)	11.069 (1.336)	15.249 (3.854)
6	39.125 (3.679)	24.570 (2.255)	33.182 (6.294)
12	68.389 (8.114)	52.786 (6.078)	58.243 (7.427)
24	123.050 (9.694)	113.074 (9.265)	90.564 (10.983)
30	137.584 (8.941)	158.990 (11.465)	109.396 (8.735)
48	198.037 (12.545)	222.613 (21.350)	142.015 (13.680)
54	204.250 (17.752)	250.487 (19.682)	163.336 (15.570)
72	254.903 (25.439)	296.408 (23.843)	187.826 (18.438)

Standard deviations of means in parentheses.

TABLE XXXV: Mean drug mass permeating human stratum corneum from ointment formulations at 35 °C and 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation		
	Betnovate	Celestoderm-V	Persivate
24	0.915 (0.346)	1.869 (1.164)	1.544 (0.956)
48	1.847 (0.146)	3.920 (2.482)	3.656 (1.793)
72	2.936 (0.758)	6.320 (3.506)	5.568 (1.860)
96	4.091 (1.050)	9.344 (4.004)	8.965 (3.172)
120	5.162 (1.483)	11.645 (5.123)	11.685 (3.759)
144	6.450 (1.553)	14.594 (5.703)	14.113 (4.742)
168	7.724 (1.647)	17.479 (6.308)	16.725 (5.106)
192	9.283 (1.283)	20.561 (6.882)	19.456 (6.364)

Standard deviations of means in parentheses.

TABLE XXXVI: Mean drug mass permeating weanling pig stratum corneum from ointment formulations at 35 °C and 600 rpm. (μg cm^{-2}).

Time (hours)	Commercial preparation		
	Betnovate	Celestoderm-V	Persivate
24	1.014 (0.114)	1.065 (0.158)	2.229 (0.434)
48	2.012 (0.182)	2.113 (0.209)	4.330 (1.918)
72	2.198 (0.021)	2.648 (0.164)	6.467 (0.978)
96	2.524 (0.092)	3.239 (0.550)	7.874 (1.093)
120	3.454 (0.933)	4.278 (1.466)	9.950 (1.492)
144	4.402 (2.062)	5.665 (2.283)	12.304 (1.845)
168	4.865 (2.424)	6.533 (3.578)	13.871 (1.324)
192	4.922 (2.420)	7.478 (4.773)	14.485 (1.448)

Standard deviations of means in parentheses.

TABLE XXXVII: Mean drug mass permeating hairless mouse skin from ointment formulations at 35 °C & 600 rpm.(μg cm^{-2}).

Time (hours)	Commercial preparation		
	Betnovate	Celestoderm-V	Persivate
16	1.427 (0.348)	2.116 (0.130)	6.416 (0.787)
24	2.957 (0.438)	4.333 (1.681)	8.228 (1.801)
48	4.403 (0.451)	9.310 (4.141)	13.109 (2.212)
72	4.742 (0.798)	14.393 (4.139)	21.479 (1.765)
96	6.080 (0.447)	21.423 (8.030)	29.616 (4.421)
120	7.327 (0.565)	23.846 (4.602)	36.025 (9.125)
144	8.360 (0.983)	31.426 (9.227)	40.610 (10.619)
168	9.349 (1.435)	36.754 (9.562)	48.130 (12.478)
192	10.391 (2.234)	42.575 (11.118)	52.339 (16.027)

Standard deviations of means in parentheses.

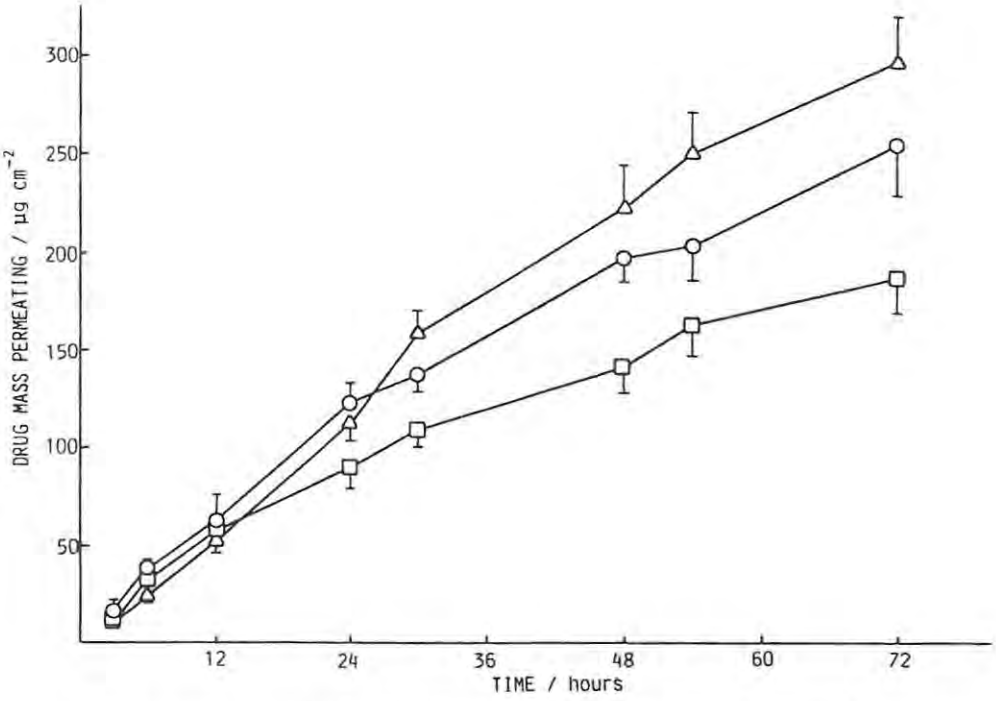


FIGURE 41: Permeation of betamethasone 17-valerate through silicone membrane from commercial ointments at 600 rpm agitation and 35 °C. ○ Betnovate, △ Celestoderm-V, □ Persivate.

Corticosteroid permeation rates are greater from the Betnovate formulation than either Persivate or Celestoderm-V, respectively, at early sampling times, whereas Celestoderm-V ointment appears to release the drug to the greater extent after 24 hours. Permeation is lowest from the Persivate formulation after the 12-hour sampling time. Statistically, the mean values from all three products are equivalent at the 1.5- and 3-hour sampling times. The Betnovate and Persivate data are statistically equivalent at the 6- and 12-hour sampling periods, as is the Persivate and Celestoderm-V data at the latter time. Furthermore, the large standard deviations of the data means make the Celestoderm-V and Betnovate formulation values statistically equivalent at the 24-, 48- and 72-hour sampling times. These values would tend to suggest that the drug permeation from Celestoderm-V and Betnovate ointments are equivalent, and that the release from these two formulations is superior to that from Persivate ointment.

The rank order of drug permeation through silicone is not paralleled by the data from any of the animal membranes. Furthermore, the biological membranes all exhibit fairly linear permeation profiles implying that the intrinsic resistivities of the media are functional in limiting the rate of corticosteroid transfer. Permeation through human stratum corneum from the ointments is approximately twice that from the cream dosage forms, and half that from the lotions. Thus, it may be proposed that drug partitioning from the different dosage forms into the stratum corneum follows the same rank

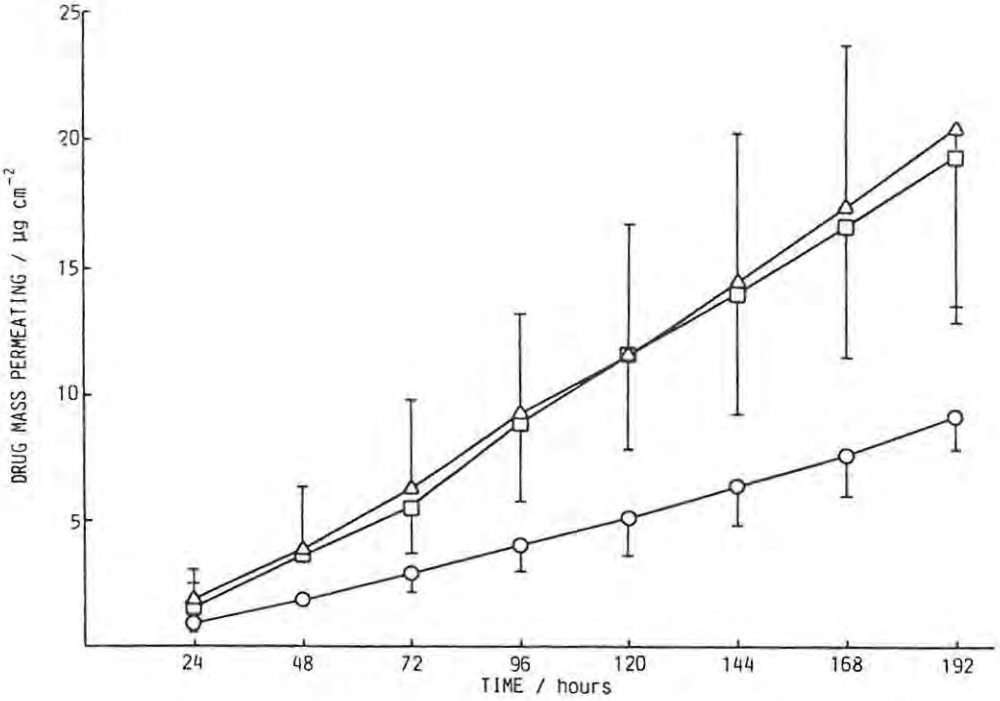


FIGURE 42: Permeation of betamethasone 17-valerate through human stratum corneum from commercial ointments at 600 rpm agitation and 35 °C.
○ Betnovate, △ Celestoderm-V, □ Persivate.

order, being greatest for lotions and lowest for the creams. Throughout the entire sampling interval the data values for the Celestoderm-V and Persivate formulations are statistically equivalent and, furthermore, are similar in magnitude and variance. This would suggest that these two formulations are equivalent in their ability to release this corticosteroid to human stratum corneum. On the other hand, permeation from Betnovate ointment is significantly less than from either of the other two formulations, suggesting relatively lower drug release capabilities for this product. This suggestion is supported by statistical analysis which demonstrates significantly lower permeation rates from the Betnovate ointment at sampling times later than 72 hours. At the 72-hour sampling time the means of the Betnovate and Celestoderm-V formulations are statistically equivalent, whereas the permeation means of all three ointments are not significantly different at the 24- and 48-hour periods.

Betamethasone 17-valerate permeating weanling pig stratum corneum is significantly greater from the Persivate ointment than from either the Celestoderm-V or Betnovate products. There are no instances of statistical equivalence between the Persivate data means and those of the other products at any sampling time. Drug permeation from Celestoderm-V ointment is slightly greater than that from the Betnovate dosage form, although statistical equivalence is demonstrated at the 24-, 48-, 96- and 192-hour sampling times. The permeation profiles from all three ointments are essentially linear and

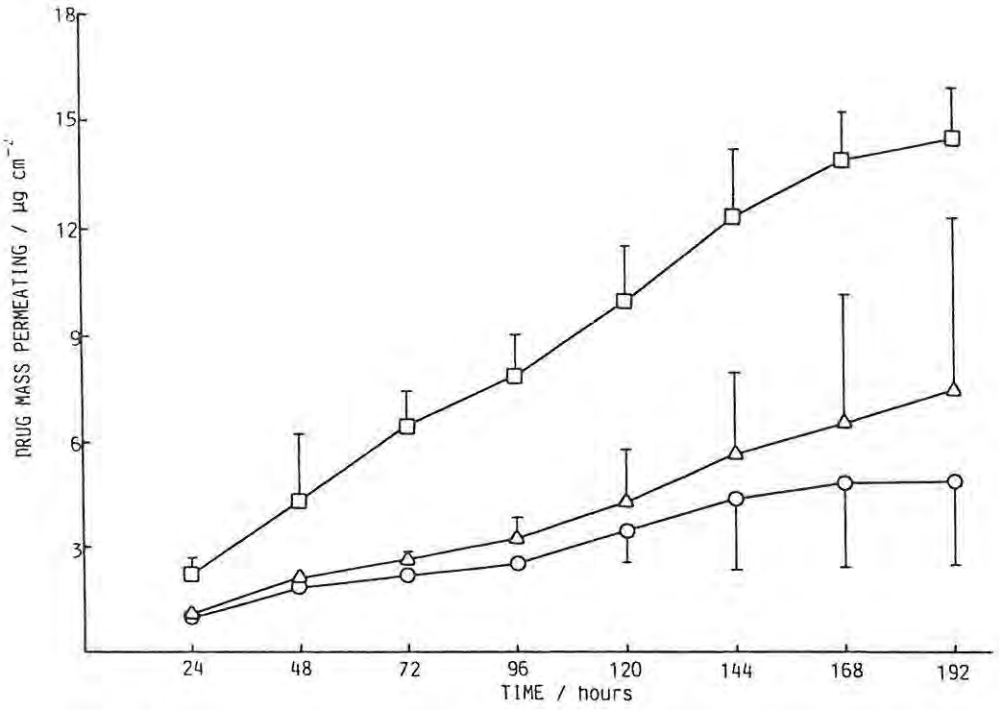


FIGURE 43: Permeation of betamethasone 17-valerate through weanling pig stratum corneum from commercial ointments at 600 rpm agitation and 35 °C. ○ Betnovate, △ Celestoderm-V, □ Persivate.

the magnitude of corticosteroid transfer over this experimental period is similar from the cream, lotion and ointment formulations. The steady-state permeation experiments of Section 4.2 have demonstrated that weanling pig membrane is more permeable to betamethasone 17-valerate passage than human stratum corneum, and yet the porcine medium does not exhibit the same variability in the rate of drug translocation from the cream, lotion or ointment formulations as is shown by the human tissue. Furthermore, the hairless mouse skin, shown to be more permeable than the weanling pig medium, also demonstrates significant variability in the drug permeation rates from these different formulations. If the porcine medium had been the least permeable of the membranes, then the drug transfer process may have attained saturation with all three dosage forms. However, the results from the steady-state experiments render this postulation inapplicable. Alternatively, when considering the human and murine membranes, it has been suggested that differential degrees of drug partitioning from the dosage forms are responsible for the different permeation rates, however it seems dubious that the biochemical composition of the pig stratum corneum could be so dissimilar to that of the other membranes to alter the drug partitioning kinetics in such a manner.

Drug permeation through hairless mouse skin follows the same rank order as that through the weanling pig stratum corneum, being greatest from Persivate ointment and lowest from the Betnovate formulation. However, the

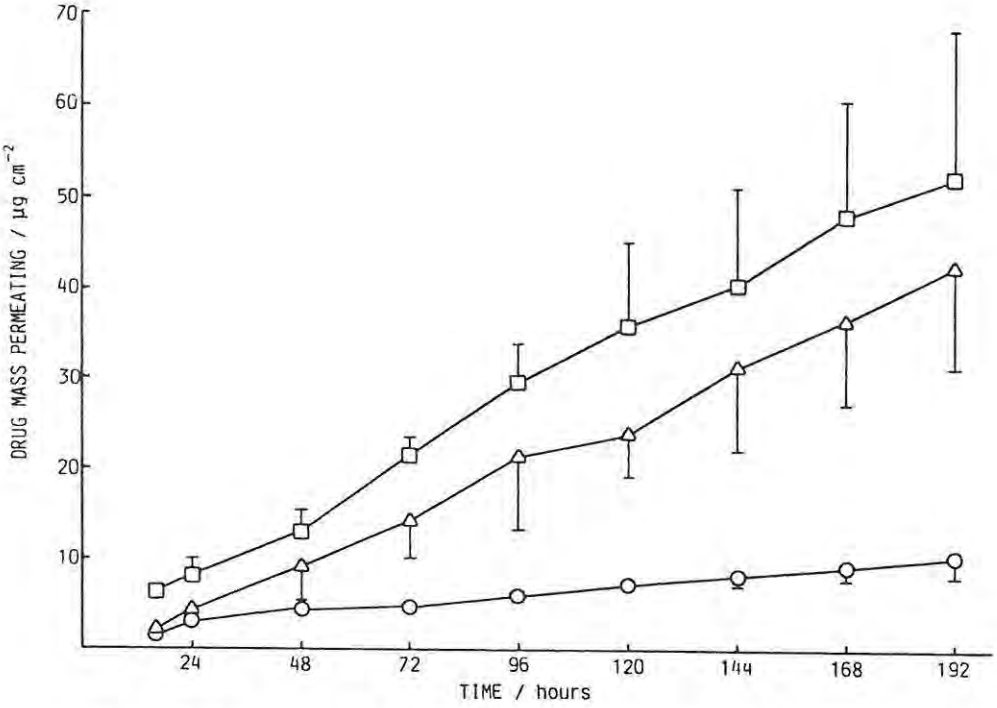


FIGURE 44: Permeation of betamethasone 17-valerate through full thickness hairless mouse skin from commercial ointments at 600 rpm agitation and 35 °C. ○ Betnovate, △ Celestoderm-V, □ Persivate.

drug permeation rates from the Persivate and Celestoderm-V formulations are not statistically different at the 48-hour and all later sampling times. This is mainly due to the overlap of the standard deviations of the means from each plot, as the actual data points do not approach each other that closely. Drug permeation from the Celestoderm-V and Betnovate formulations are not significantly different at the 16-, 24- and 48-hour sampling times, but thereafter there is significant difference between the two profiles.

Corticosteroid transfer from the ointment formulations through hairless mouse skin is slightly greater than that from the creams, but is approximately half that from the lotion dosage forms. In this respect the human and murine membranes are alike. These differential flux rates may be explained, as before, by reference to preferential drug partitioning from the lotions, and to a lesser extent from the ointment environments.

In summary, drug permeation from ointment dosage forms through animal and synthetic membranes are dissimilar in that linear profiles are obtained for the animal tissues whereas a decrease in flux through silicone membrane is observed with time. With all three animal membranes the drug permeation from Betnovate ointment is markedly the slowest, whereas this formulation occupies an intermediate ranking with the silicone membrane. Corticosteroid permeation is fastest from Persivate ointment through weanling pig and hairless mouse membranes, and is equivalent to that from Celestoderm-V

ointment through human stratum corneum. On the other hand, permeation is slowest from Persivate through the silicone medium.

Therefore, it is apparent that the membranes are all able to discriminate between the commercial ointments with respect to their drug release potential. However, while the results from the three animal membranes are markedly similar, correlation of the results from the animal membranes with those from the silicone medium is not demonstrated, and, furthermore, a reversal of the rank permeation order is observed.

SCALP APPLICATION

Corticosteroid permeation from Betnovate scalp application, a 0.1% solution of betamethasone (as the 17-valerate) in an alcoholic base, was tested by *in vivo*-mimic methodology. This was the only commercial formulation of its kind available on the South African market. Permeation was measured at 35 °C from infinite donor doses of this formulation through human and weanling pig stratum corneum, and full thickness hairless mouse skin, to a receptor solvent of purified isopropyl myristate. The data from these experiments are presented in Table XXXVIII, and plotted in Figure 45.

Preliminary investigation using silicone membrane indicated that this medium presents a negligible barrier to the passage of the corticosteroid from the alcoholic dosage form. The average permeant masses measured in the receptor chamber after one, three and six hours of experimentation using this membrane were 56.615, 272.950 and 395.588 μg cm^{-2}, respectively. These values are plotted in Figure 45 for comparison. In view of this flux magnitude, further monitoring of permeation through silicone membrane was abandoned as the profile obtained here clearly does not correlate with that from any of the animal membranes, or from those of the previously reported dosage forms.

TABLE XXXVIII: Mean drug mass permeating human and weanling pig stratum corneum, and full thickness hairless mouse skin from Betnovate scalp application at 35 °C and 600 rpm. (μg cm^{-2}).

Time	Membrane		
(hours)	Human[a]	Weanling pig[a]	Hairless mouse[b]
4-6	2.959 (1.172)	2.144 (0.665)	2.265 (0.589)
16	6.530 (2.272)	4.467 (0.540)	10.010 (1.824)
24	10.398 (3.619)	5.566 (0.912)	23.196 (7.507)
30	14.765 (3.964)	10.084 (2.404)	43.360 (12.139)
48	35.872 (7.805)	14.054 (1.932)	73.541 (18.697)
60	56.633 (17.625)	26.994 (0.899)	166.467 (25.070)
72	97.833 (21.285)	39.533 (6.908)	238.462 (50.171)
84	159.184 (23.834)	55.699 (4.108)	377.533 (55.512)
96	212.359 (50.535)	68.166 (5.714)	517.516 (122.677)

Standard deviations of means in parentheses.
[a] stratum corneum, [b] full thickness skin.

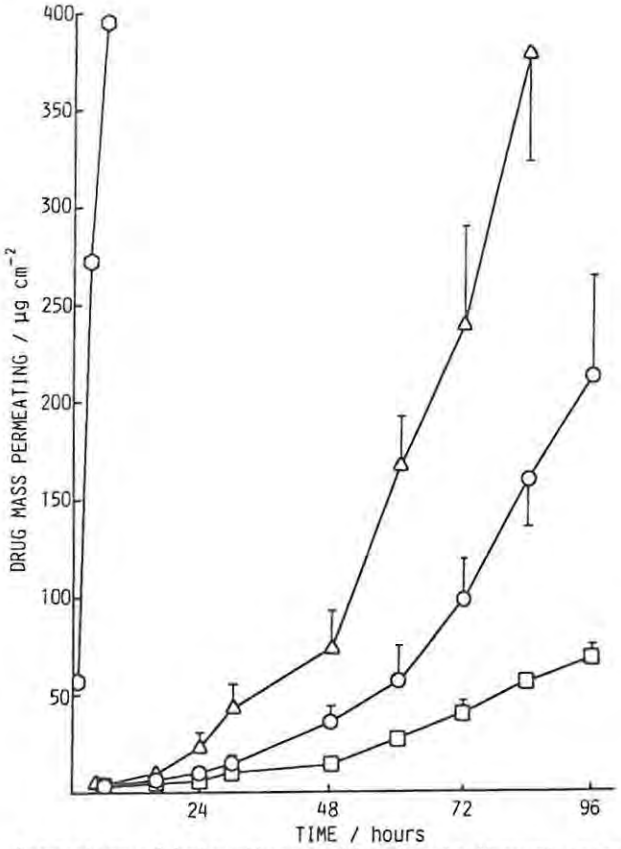


FIGURE 45: Permeation of betamethasone 17-valerate through animal and synthetic membranes from Betnovate scalp application at 600 rpm agitation and 35 °C. ○ human stratum corneum, △ full thickness hairless mouse skin, ○ silicone membrane, □ weanling pig stratum corneum.

The permeation profiles of betamethasone 17-valerate through the animal membranes from the alcoholic scalp application do not resemble those from any of the other dosage forms. Here, an exponential increase in the drug flux rate is observed with time through hairless mouse and human media and, to a lesser extent, through weanling pig stratum corneum. Statistically the mean data values for all three animal membranes are equivalent at the first sampling time, and equivalence is demonstrated between the permeation through human and weanling pig media at the 16- and 30-hour sampling periods. At all other times there is significant difference between the permeation means. These observations are in direct contrast to the linear or decreasing flux profiles previously described from the creams, lotions and ointments. The drug flux rate from the scalp application at 96 hours is approximately 32-times greater than that from the ointments, 20-times greater than that from the creams, and 12-times greater than that from the lotion dosage forms at equivalent sampling times.

The contrast to previously reported results is even more marked when one considers the silicone membrane. Drug permeation from the application only appears to be hindered slightly by this medium and exceptionally high permeant masses are detected in the receptor chamber after only six hours. The permeation rates at this time are approximately 87-, 56- and 20-times greater

than those from the ointments, creams and lotions, respectively. Moreover, the drug permeability coefficient for this membrane using the donor scalp application is threefold greater than that recorded in the steady-state diffusion experiments (Section 4.2) using a 0.1% solution of betamethasone 17-valerate in isopropyl myristate as the donor vehicle.

It is obvious, therefore, that drug release or partitioning from the scalp application is significantly different to that from the other semi-solid dosage forms or the isopropyl myristate solution studied. It is possible that the alcoholic base of the application modifies the interchain, solvent environment of the silicone polymer in such a manner that drug passage is facilitated. The alcoholic molecules may act in a surfactant-like manner, interacting with the polar and nonpolar groups of the silicone polymer chains to reduce the surface tension within this region. Furthermore, the alcohol greatly augments the solvent environment for the corticosteroid within the membrane, thereby facilitating drug translocation.

On the other hand, it seems feasible that prolonged contact (several days) of the animal membranes with the alcoholic solution may denature the biochemical composition of these media to such an extent that drug passage becomes more facile with time. While several reports in the literature have indicated that short periods (hours) of alcoholic contact do not impair the barrier properties of prepared animal membranes significantly *(24-27)* in this case it is proposed that immersion for several days at 35 °C may solubilize lipids or proteins within the barrier strata and these compounds could then be passed, in solution, into the donor or receptor phases. This postulation is supported by examination of Figure 45 which indicates that essentially linear permeation profiles are detected for all three animal membranes up to approximately the 48-hour sampling time, and possibly the 60-hour sampling time for the human and weanling pig media. Thereafter, marked increases in the permeation rates are observed in typically exponential fashion, especially for the hairless mouse and human membranes.

If one assumes that biogenic components are being extracted from the barrier stratum corneum by prolonged alcoholic contact, then it is interesting to note that the degree of extraction follows the rank order thickness of these membranes. Extraction of compounds, as measured by the relative increase in drug permeation with time, is greatest for the hairless mouse skin, which also has the thinnest stratum corneum and therefore, presumably, the smallest total quantity of extractable lipids or proteins. Depletion of this relatively thin layer may therefore be extensive over the course of the experiment. Conversely, weanling pig stratum corneum is the thickest barrier

medium studied and demonstrates the smallest relative increase in permeation with time, the compound extraction here being proportionately less than for the other membranes. Human stratum corneum has a thickness intermediate between hairless mouse and weanling pig and also demonstrates an intermediate increase in permeation rate with time. Therefore, while the same absolute quantity of lipids and proteins may be solubilized and extracted from each of the membrane types per unit time, this quantity may represent a large percentage of the barrier potential of the hairless mouse skin, an intermediate percentage of the compounds forming the human medium barrier, and a relatively small percentage of the pig stratum corneum barrier.

SUMMARY

From the foregoing results it is apparent that no two membranes perform identically under the same experimental conditions, although in a number of cases the animal media produce similar, correlating results. In most cases the drug masses permeating the different membranes under identical experimental conditions are of different magnitudes and, thus, where correlation is observed between the results of two membranes this is usually in rank order of permeation from the different formulations, rather than in terms of absolute permeant masses. In certain cases rank order agreement is also demonstrated between the permeation results from the animal and silicone membranes. A tabulated comparison of the rank order permeation for the different membranes and dosage forms is presented in Table L, Section 4.5. Permeation profiles for the silicone medium clearly follow the square-root-of-time relationship for the cream, lotion and ointment formulations, whereas, of the animal media, this relationship is only observed with the human stratum corneum and lotion dosage form. The majority of the other permeation profiles may be regarded as linear, except for the scalp application which shows exponential increase in flux rate through the animal media with time. An examination of the average permeability coefficients for betamethasone 17-valerate from the different topical dosage forms is presented in Table XXXIX. In comparison, the steady-state betamethasone 17-valerate permeability coefficients from the 0.1% solution in isopropyl myristate (Section 4.2) for silicone membrane, human and weanling pig stratum corneum, and hairless mouse skin are 221.181-, 1.273-, 2.743- and 14.882 x 10^{-4} cm h^{-1}, respectively. The drug permeability coefficients for hairless mouse skin from the extemporan-eously prepared buffered cream without enhancer, 2%-resorcinol cream, 5%-resorcinol cream and 10%-urea cream are 4.680-, 8.247-, 15.420- and 4.936 x 10^{-4} cm h^{-1},

respectively.

TABLE XXXIX: Average permeability coefficients for betamethasone 17-valerate from commercial topical formulations. ($cm\ h^{-1} \times 10^4$).

Formulation	Membrane	Commercial dosage form		
		Betnovate	Celestoderm-V	Persivate
Cream	Silicone	16.157	14.877	21.126
	Human	0.466	0.507	0.729
	Weanling pig	0.420	0.593	0.758
	Mouse	1.734	1.765	1.591
Lotion	Silicone	23.632	25.579	-
	Human	2.758	2.641	-
	Weanling pig	0.571	0.703	-
	Mouse	4.875	4.515	-
Ointment	Silicone	50.962	44.306	41.606
	Human	0.428	0.943	0.884
	Weanling pig	0.318	0.390	0.852
	Mouse	0.736	1.986	3.073
Scalp application	Silicone	711.766	-	-
	Human	9.982	-	-
	Weanling pig	4.431	-	-
	Mouse	23.384	-	-

- Dosage forms unavailable on South African market.

The corticosteroid permeability coefficients measured from the commercial semisolid formulations through silicone membrane are approximately 5- to 14-times smaller than the coefficient measured from the 0.1% drug solution in isopropyl myristate. This would imply that a combination of drug release and partitioning between formulation and polymer is governing the rate of permeation. Linear permeation mass *versus* square-root-of-time plots indicate that drug release from the formulation may be the more prominent factor. Conversely, the permeability coefficient measured from the scalp application is over threefold greater than that from the isopropyl myristate solution. A greater interaction of the alcoholic dosage form constituents with the silicone medium is postulated to be the cause of this greater flux magnitude.

Permeability coefficients measured from the commercial cream and ointment formulations through human stratum corneum are approximately half that measured from the isopropyl myristate solution, whereas, unique to the animal media, the coefficient measured from the lotion is twice that from this solution. Furthermore, the drug mass permeating from the lotion also displays linearity with the square-root-of-time. The partition coefficient of the drug between the lotion and membrane is obviously highly favourable, moreso than that from isopropyl myristate solution, and results in rapid drug depletion at the membrane interface.

The commercial cream, lotion and ointment permeability coefficients measured through weanling pig stratum corneum and hairless mouse skin are all at least three times smaller than the coefficients measured in the steady-state experiments using the 0.1% drug donor solution. From the linearity of the profiles obtained with these media, and with drug permeation through human

stratum corneum from cream and ointment formulations, it is assumed that the partition coefficient is dominant in governing the flux rate. Conversely, drug permeability coefficients through hairless mouse skin from the extemporaneously prepared creams are, at least, approximately three times and, at most, tenfold greater than the coefficients from the commercial cream formulations. Furthermore, the permeability coefficient from the cream containing 5% resorcinol is greater than that from the steady-state diffusion experiments using isopropyl myristate drug solution. These data further exemplify the superior drug partitioning between the extemporaneous cream formulations and hairless mouse skin.

With all three animal media, permeability coefficients from the scalp application are at least double that observed from the isopropyl myristate solution. The increase in permeability for each membrane is ascribed to abstraction of lipid or protein constituents from the barrier stratum corneum.

4.4 RESULTS FROM *IN VIVO* BLANCHING ASSAYS

Human blanching bioassays were conducted using the same batches of topical corticosteroid formulations that were assessed by *in vitro* methodology and reported in Section 4.3. In this fashion the drug bioavailability *in vivo* and rank order of permeation through living skin could be compared to the results obtained *in vitro* using excised skin or synthetic membrane. The blanching trials were conducted as detailed in Section 3.6, any deviation from this general protocol, where applicable, is recorded below under the description of each trial. Briefly, specific quantities of the different formulations were applied and spread over discrete sites on the forearms of the volunteers. These sites were then either left open to the atmosphere (unoccluded mode) or covered with impervious tape (occluded mode) for six hours, after which any residual formulation was removed. The degree of blanching induced in the skin was then subjectively assessed by three observers over several hours to yield a blanching profile. A comparison of the blanching elicited by different formulations applied to neighbouring sites was also made. The results for each preparation are reported as the percentage of the total possible score (%TPS) that the formulation could have achieved *versus* the time after application and these percentages are used to calculate the area under the curve (AUC) value. The profiles so generated give an estimation of the extent and duration of the betamethasone 17-valerate permeation. The statistical significance of the difference between profiles

profiles has been assessed by chi-squared analysis. Along with the full experimental details, Section 3.6 also has a tabulated summary of the products tested and the application modes used in each blanching trial.

COMMERCIAL CREAMS

Nine volunteers were used to assess the blanching responses to the three commercial cream formulations (Betnovate, Celestoderm-V and Persivate), each containing a label concentration of 0.1% betamethasone (as the 17-valerate), applied in the unoccluded mode. The results from this assay are presented in Table XL and plotted in Figure 46.

TABLE XL: Blanching response to commercial betamethasone 17-valerate cream formulations applied in the unoccluded mode. (%TPS).

Time (hours)	Commercial formulation		
	Betnovate	Celestoderm-V	Persivate
7	28.935	24.769	27.315
8	34.491	29.398	33.796
9	40.741	37.037	42.824
10	47.454	40.509	47.454
12	52.546	46.759	59.259
14	55.324	49.074	64.583
16	47.685	41.435	57.870
18	41.435	36.343	53.704
28	18.056	16.435	26.157
32	19.444	16.898	29.167
AUC	987.153	867.708	1184.144

TABLE XLI: Blanching response to lotion formulations, unoccluded mode.(%TPS).

Time (hours)	Commercial formulation	
	Betnovate	Celestoderm-V
7	14.699	13.889
8	21.181	20.602
9	26.273	27.894
10	31.713	31.019
12	40.394	39.815
14	42.014	41.088
16	38.079	39.005
18	32.639	32.986
28	21.991	22.569
AUC	700.579	701.157

The rank order of blanching generated by the commercial cream formulations over the 32-hour interval is Persivate greater than Betnovate, which was greater than Celestoderm-V. Based on this rank order, it is proposed that Persivate cream is best able to release its corticosteroid to the human stratum corneum, release is slightly lower from Betnovate cream, and Celestoderm-V has the, relatively, lowest drug release potential. It must be borne in mind that this order was obtained from the unoccluded mode of application and occasionally a reversal of trends is observed if the same formulations are tested in the occluded mode. The hydrating effect of occlusion is expected to produce a faster onset of blanching, a greater peak value and a longer duration of action for each formulation.

Statistically, examination of the graded response values demonstrates significant difference between the Persivate and Celestoderm-V observations at the 12-hour and later reading times. Moreover, comparison of the blanching at neighbouring sites to which these two preparations had been applied yields significant chi-squared values at every reading time. This would imply that the differences in the drug release rates from these two formulations in the

unoccluded application mode are statistically real.

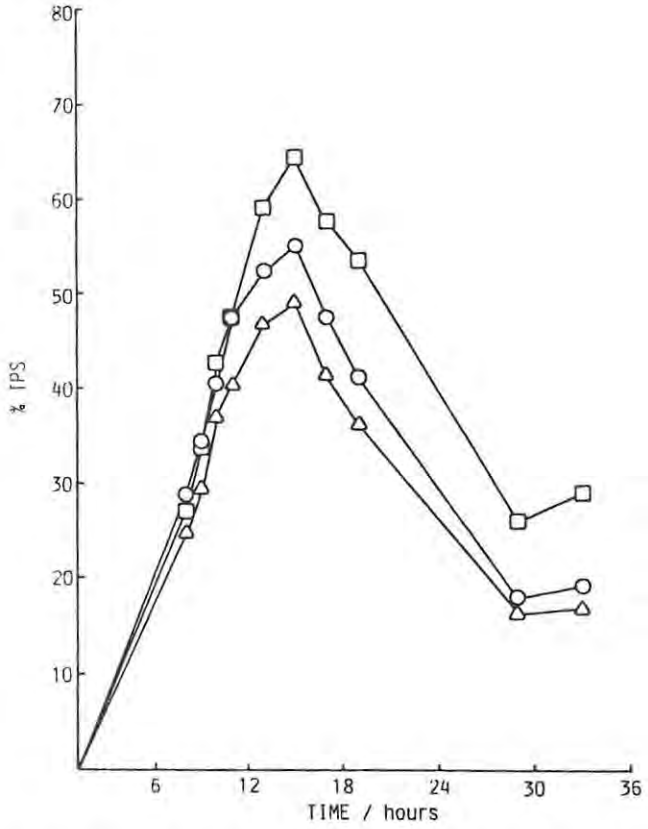


FIGURE 46: Blanching profiles of commercial betamethasone 17-valerate creams
○ Betnovate, △ Celestoderm-V, □ Persivate. Unoccluded mode.

The graded response values recorded for the Persivate cream are significantly different to those of the Betnovate formulation at reading times between 16 and 32 hours, however the difference in blanching appears, from the graph, to be equally large at the previous 14-hour reading. Here the variance between the readings from the three observers obviously diminishes the significance of the difference to below the 95% level. On the other hand, the neighbouring-site comparisons between Persivate and Betnovate creams show significant differences at the early reading times (at the 7-, 8- and 12-hour periods). Hence the two statistical tests applied to the data demonstrate significant difference between the Persivate and Betnovate blanching at early and late readings, with equivalence at the profile apex.

The statistical comparison of graded response values for the Betnovate and Celestoderm-V creams produced no incidence of significant difference between the blanching values of the two preparations. However, the neighbouring-site comparisons yielded significant difference between the two blanching responses at the 28- and 32-hour periods. Essentially, therefore, there is statistical equivalence of these two blanching profiles even though the rank order of the Betnovate is greater than that of the Celestoderm-V cream.

These blanching results may be compared to those from similar investigations conducted in our laboratories. The batches of the commercial products used by the other researchers differ from those of the present study and, hence, it cannot be guaranteed that the manufacturers have not modified the product formulations between the time the previous and present studies were conducted. For the purposes of this comparison it will be assumed that the various product formulae have not changed and, thus, different batches of the same formulation have been assessed for blanching potential in each case.

Meyer *(272)* observed a similar rank order of blanching for the creams in the unoccluded mode, only in this case the profile of the Persivate cream was much greater than that of either Celestoderm-V or Betnovate, the profiles of the latter two preparations being essentially equivalent. However, testing the same creams in the occluded application mode produced a greater AUC value for Celestoderm-V cream than either Persivate or Betnovate, in that order. The blanching of the Celestoderm-V was statistically greater than that of the Persivate when assessed by the adjacent site comparison but was equivalent when assessed by the graded response comparison. On the other hand, Coleman *(290)* has observed essentially coincident blanching profiles for Betnovate and Celestoderm-V creams in both modes of application, the blanching produced by Persivate cream was not assessed in this case.

In summary, the blanching elicited by Persivate cream in the present investigation appears to be superior, in rank order and statistical significance, to that of either Betnovate or Celestoderm-V creams. The rank order of the blanching from the Betnovate product is greater than that from Celestoderm-V cream, however these differences are not statistically significant.

LOTIONS

Twelve volunteers were used to assess the blanching responses to the two commercial lotion formulations (Betnovate and Celestoderm-V), each containing a label concentration of 0.1% betamethasone (as the 17-valerate), applied in the unoccluded mode. The results from this assay are presented in Table XLI and plotted in Figure 47.

The similar blanching profiles and AUC values obtained in this assay indicate that the degree of drug release from the two formulations is equivalent at all observation times. The Betnovate lotion produces a slightly greater rank order of blanching than the Celestoderm-V product at the 12- and 14-hour reading times. At earlier times there is no clear rank order

preparations were essentially equivalent: in the unoccluded application mode the AUC value for Celestoderm-V lotion was found to be marginally less than that for Betnovate while the reverse situation was observed in the occluded mode. In all cases there were no significant statistical differences between the data values of the two blanching profiles. Furthermore, in support of the previous observation, Meyer also reports that the maximum blanching elicited by the lotions in the unoccluded mode is less than that observed for the other formulation types, however this relatively lower response for the lotions was not observed in the occluded application mode.

It would appear, therefore, from a number of studies undertaken, that the two lotion formulations are equally able to release their drug content to the stratum corneum. The different statistical tests applied to the data do not show any significant difference between the responses of the two formulations at any observation time.

OINTMENTS

Nine volunteers were used to assess the blanching responses to the three commercial ointment formulations (Betnovate, Celestoderm-V and Persivate), each containing a label concentration of 0.1% betamethasone (as the 17-valerate), applied in the unoccluded mode. The results from this assay are presented in Table XLII and plotted in Figure 48.

TABLE XLII: Blanching response to commercial betamethasone 17-valerate ointment formulations applied in the unoccluded mode. (%TPS).

Time (hours)	Commercial formulation		
	Betnovate	Celestoderm-V	Persivate
7	17.824	21.759	34.954
8	20.602	28.704	40.046
9	26.157	39.120	47.685
10	31.019	43.750	53.935
12	39.120	53.935	65.278
14	41.435	55.324	73.380
16	37.732	51.157	69.444
18	34.722	43.287	65.741
28	15.509	19.444	35.185
32	15.972	17.824	31.944
AUC	750.000	972.801	1429.282

The three blanching profiles generated by the commercial ointment formulations differ markedly from one another compared to the profiles from the cream formulations. Here the blanching elicited by the Persivate ointment is clearly superior to that of Celestoderm-V cream, which, in turn, is superior to that of the Betnovate formulation. Significant statistical difference

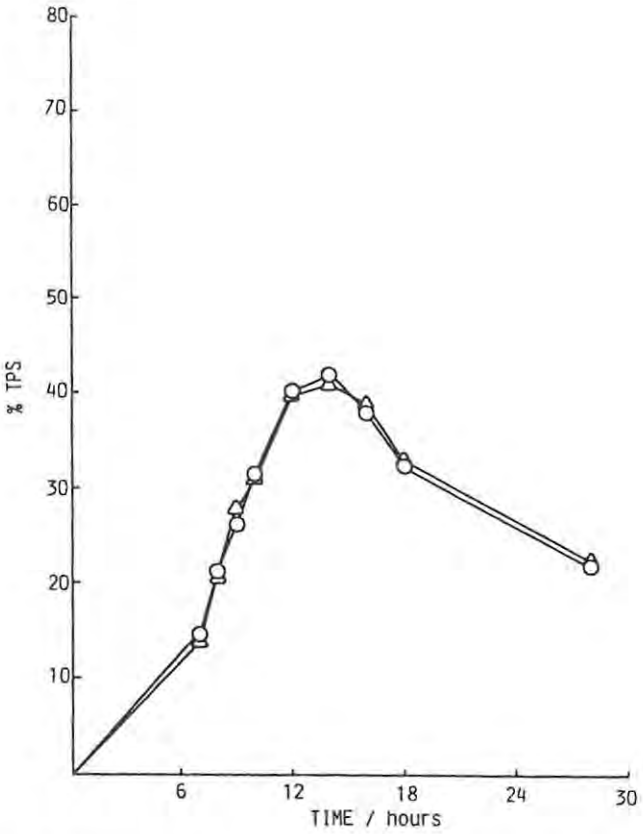


FIGURE 47: Blanching profiles of commercial betamethasone 17-valerate lotions assessed in the unoccluded application mode.
○ Betnovate, △ Celestoderm-V.

superiority of the blanching from one product over that from the other, whereas during the latter interval of the assay the blanching induced by Celestoderm-V is slightly greater than that elicited by Betnovate lotion. Chi-squared statistical evaluation of the data, by either graded response or comparison of blanching at adjacent sites, indicates that the recorded values at all observation times are not significantly different, as would be expected from the very similar profiles.

It is interesting to note that the maximum %TPS attained by the lotion formulations is approximately 45%, this is only 60% of the maximum value attained by the cream or ointment formulations. However, the three trials were conducted on different days, using different volunteers and hence conclusions should be drawn from these values with caution. At face value these maxima suggest that drug release from the lotions formulations is lower than that from the other semisolid formulations. This may possibly be due to the presence of solubilizing agents, such as propylene glycol, in the lotions. The corticosteroids have high affinity for these agents and, hence, their tendency to partition into the skin is diminished.

Meyer *(272)* has reported identical results from similar blanching assays undertaken in both application modes. The profiles reported for the two

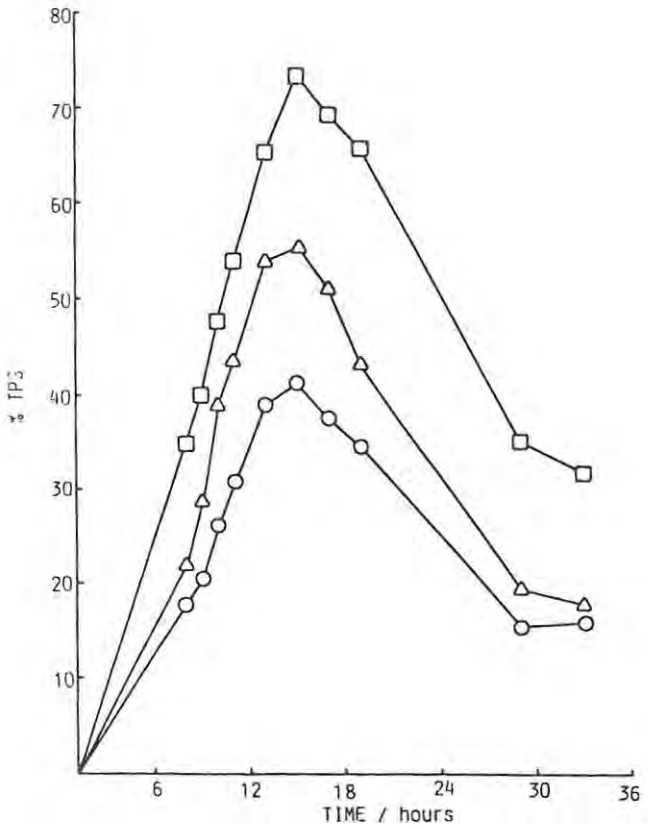


FIGURE 48: Blanching profiles of commercial betamethasone 17-valerate ointments assessed in the unoccluded application mode.
○ Betnovate, △ Celestoderm-V, □ Persivate.

between the three blanching profiles, based on the graded response values, is exhibited at all periods between the 8- and 16-hour reading times, inclusive. At the 7-, 18-, 28- and 32-hour reading periods there is significant difference between the Persivate values and those of both the Celestoderm-V and Betnovate formulations, but no difference between the readings of the latter two preparations. Hence, the differences between the blanching data of the Celestoderm-V and Betnovate preparations are not significantly different at the early and late reading times, but are significantly different during the period of maximum observed blanching (approximately 14 hours). Similar statistical results are obtained for the three preparations from the comparison of blanching at adjacent sites. These data indicate that the differences in the corticosteroid release profiles are statistically real.

In conducting similar experiments, Meyer *(272)* has observed that Persivate ointment releases the greatest amount of corticosteroid in both the occluded and unoccluded modes of application. In the unoccluded assay Betnovate ointment demonstrated rank superiority over Celestoderm-V ointment in the profiles generated, although these differences were not statistically significant, whereas in the occluded assay the profiles from these two preparations were equivalent statistically and by rank order. In contrast to the

pipette. From this data the mass or volume of each formulation to be applied could be calculated so that an equivalent mass of betamethasone 17-valerate was spread over each application site.

Each formulation was marketed at a label concentration of 0.1% betamethasone (17-valerate), the actual assay values for each product are given in Table XLIII along with the mass or volume of each formulation applied to the forearm sites and the mass of betamethasone 17-valerate contained within this application aliquot. This list indicates that the masses of drug applied for each of the formulations were not identical. This was mainly due to the difficulty in precisely extruding a fraction of a stripe of the semisolid formulations from the application syringes. Whole-stripe values were therefore adopted for the cream and ointment and the volumes of the lotion and scalp application were adjusted to yield an average applied drug mass of 3.58 mg (SD = 0.11 mg).

TABLE XLIII: Betnovate formulation assay values, application aliquots and mass of betamethasone 17-valerate applied to each site.

Formulation	Percentage purity	Aliquots applied	Mass (mg)
Cream	99.02	3 stripes	3.45
Lotion	91.54	6.9μl	3.55
Ointment	104.50	5 stripes	3.70
Scalp Application	121.61	3.9μl	3.61

The degrees of blanching induced by the different formulations were assessed in both the unoccluded and occluded application modes. These results are presented in Tables XLIV and XLV, and plotted in Figures 49 and 50, respectively.

TABLE XLIV: Blanching response to commercial Betnovate formulations applied in the unoccluded mode. (%TPS).

Time (hours)	Betnovate formulation			
	Cream	Lotion	Ointment	Scalp appl.
7	25.926	27.778	27.778	38.657
8	33.333	34.954	35.417	42.824
9	40.509	36.111	43.519	50.926
10	45.138	39.815	48.148	56.250
12	49.306	43.750	53.935	61.574
14	51.157	42.361	56.250	56.019
16	50.000	41.204	53.935	53.472
18	41.667	33.102	43.750	43.519
28	15.278	14.352	12.732	17.361
32	12.963	11.343	11.343	15.509
AUC	929.051	818.287	964.815	1088.542

TABLE XLV: Blanching response to commercial Betnovate formulations applied in the occluded mode. (%TPS).

Time (hours)	Betnovate formulation			
	Cream	Lotion	Ointment	Scalp appl.
7	36.343	46.065	28.472	50.463
8	44.907	52.315	33.333	60.880
9	53.704	59.722	41.204	67.824
10	59.028	65.741	49.074	73.843
12	64.352	71.528	57.176	77.778
14	62.500	70.139	59.028	77.083
16	58.333	62.269	55.787	68.287
18	47.454	51.158	47.454	57.639
28	11.343	11.806	12.037	15.509
32	9.028	11.343	11.574	13.657
AUC	1085.069	1215.046	998.148	1369.329

The decreasing rank order of blanching in the unoccluded application mode, as indicated by the AUC values, is scalp application, ointment, cream and lotion. Having applied the same mass of betamethasone 17-valerate to each forearm site, it is interesting to note the markedly different blanching profiles elicited by the different formulations and to speculate on reasons

results of Meyer, and in congruity with the results of this investigation, Coleman *(290)* has observed the blanching profile of Celestoderm-V ointment to be superior to that of Betnovate in both application modes. Furthermore, significant differences between the two profiles are evident at early recording times, while statistical equivalence of the data is demonstrated at later periods. These observations are similar to those reported for Celestoderm-V and Betnovate ointments in this investigation.

Hence, it can be concluded that betamethasone 17-valerate release from Persivate ointment is definitely greater than that from either Celestoderm-V or Betnovate formulations at all observation times. Blanching elicited by Celestoderm-V is superior to that of Betnovate ointment over the period of maximum blanching, but the magnitudes of drug release from these two formulations are statistically equivalent at the initial and at later observation times.

BETNOVATE FORMULATIONS

Twelve volunteers were used to assess the blanching responses to four different Betnovate topical preparations (cream, lotion, ointment and scalp application) representing the four commercial products containing 0.1% betamethasone (as the 17-valerate) available on the South African market under the Betnovate tradename. The methodology for this trial varied from the general scheme outlined in Section 3.6 in that each forearm application site was exposed to an equivalent mass of corticosteroid, contained within different volumes of each formulation type. In normal assay protocol equal masses or volumes of each formulation are applied to each site. This may result in different masses of active ingredient being exposed to the skin as a result of varying drug concentrations within these formulations. It was believed that these small, interformulation concentration differences may influence the magnitude of blanching elicited, and ,in this study, it was important that only the formulation influences on drug release and subsequent blanching be manifest, without the additional influences of variable drug masses.

The products were each assayed by the high-performance liquid chromatographic method described in Section 3.1 which yielded the exact concentration of each product. Serial weighings were conducted to establish the mass of each stripe of cream or ointment extruded from the application syringe, or the mass per µl of lotion or scalp application delivered from the transfer

for relatively greater drug movement from one formulation compared to that from another.

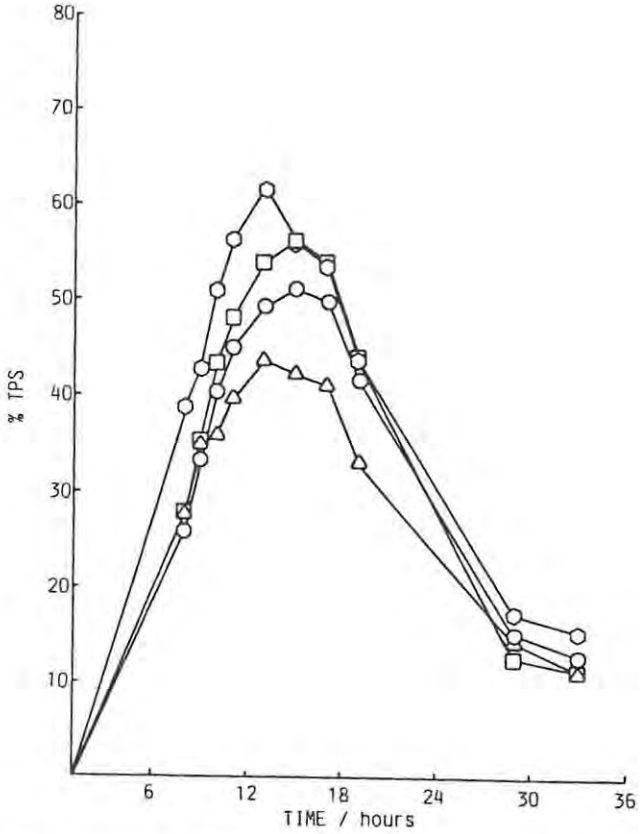


FIGURE 49: Blanching profiles of commercial Betnovate formulations assessed in the unoccluded application mode.
○ cream, △ lotion, □ ointment, ⬡ scalp application.

It was anticipated that drug release would be greatest from the alcoholic solution of the scalp application. Partitioning of the corticosteroid from this simple formulation to the stratum corneum is obviously a facile process. Furthermore, movement of the alcohol molecules into the skin may assist in transporting the drug or in modifying the environment of the horny layer so that the affinity of the drug molecules for this medium is enhanced. The inherently occlusive nature of the lipophilic ointment formulation tends to hydrate the stratum corneum, even in the unoccluded application mode, and this trapped moisture enhances corticosteroid movement through the tissue. The relatively high degree of drug permeation observed from this dosage form is also, therefore, not unexpected. The blanching generated by the cream formulation occupies an intermediate position between that of the ointment and lotion dosage forms. The creams are generally less occlusive to transpirational loss and, thus, less extensive blanching profiles are normally observed for creams than for ointments. The low degree of blanching elicited by the lotion formulation was unexpected in that it was assumed the drug would be solubilized in this relatively nonviscous formulation in the presence of cosolvents that may enhance partitioning or drug movement through the stratum

corneum. In comparison to the other products it appears that, in the unoccluded mode, the affinity of the drug for the lotion vehicle is greater than its affinity for the skin.

Statistically, the blanching produced by the scalp application is significantly superior to that of the lotion at all observation times between 7 and 18 hours, inclusive, when comparing the graded response values, and up to the 16-hour period when comparing the blanching at adjacent sites. The blanching of the scalp application is significantly superior to that of the cream between the 7- and 12-hour readings for the graded response values, and, for the adjacent site comparisons, at 7 hours and 9-12 hours. The graded response values for the scalp application are significantly greater than those of the ointment between 7 and 12 hours, and at the 18-hour observation, whereas the comparison of blanching at adjacent sites demonstrates significant differences between 8 and 10 hours, and between 28 and 32 hours.

Generally, there are significant differences between the degree of blanching elicited by the scalp application and that produced by the other formulations at the early and peak observation times, whereas differences are less significant at later periods.

A comparison of the blanching produced by the ointment and lotion formulations demonstrates significant differences between the 9- and 18-hour observations for the graded response values, and between 9 and 28 hours for the comparison of blanching at adjacent sites. The blanching differences between the products are, thus, not significant at the beginning and end of the assay. On the other hand, there is no statistical difference, by either of the statistical methods employed, between the blanching produced by the ointment from that produced by the cream at any observation time, even though there is an obvious rank order difference between these two profiles.

Comparing the blanching elicited by the cream formulation to that produced by the lotion, either by the graded response or adjacent site techniques, demonstrates significant differences between the 14- and 18-hour observation times, inclusive. At all other periods these blanching profiles are statistically equivalent.

In summary, the blanching profile of the scalp application attains its peak value approximately two hours earlier than the profiles of the other formulations. The ointment, cream and lotion produce similarly shaped profiles that peak at approximately 14 hours. The statistically significant

differences between the profile of the scalp application and those of the other formulations are, therefore, demonstrated at the early observation times, the profiles being equivalent after 16 hours. In contrast the significant differences between the profiles of the other formulations are demonstrated over the interval of peak blanching, with equivalence observed at early and late reading times.

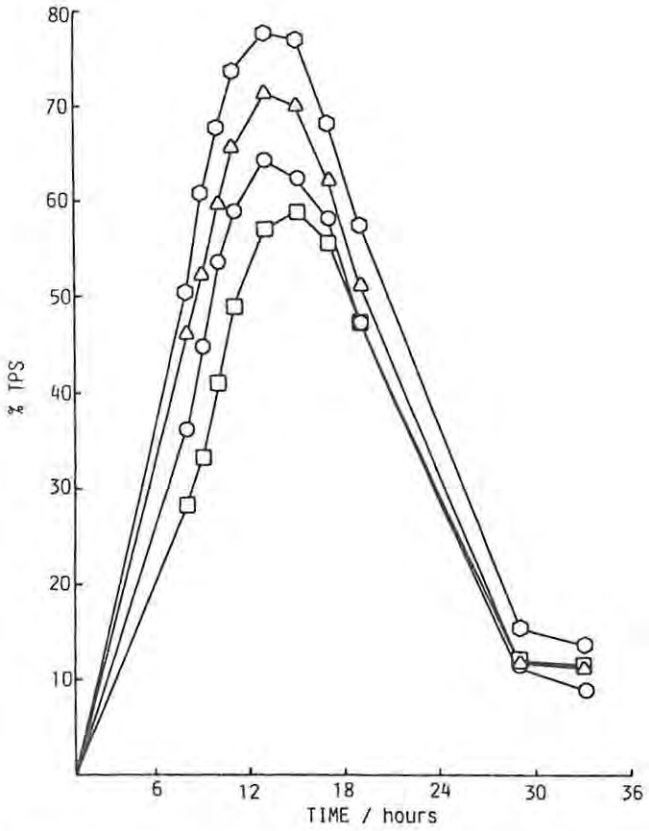


FIGURE 50: Blanching profiles of commercial Betnovate formulations assessed in the occluded application mode.
○cream, △lotion, □ointment, ○scalp application.

The use of occlusive dressings in the blanching assay increases the degree of drug permeation from all four dosage forms and causes peak blanching to be elicited 2-4 hours earlier, when compared to the unoccluded data. The most marked difference is observed for the lotion formulation which increases its peak %TPS value from 40% to approximately 70% in the occluded mode. The additional horny layer moisture that is present in this case must interact with the cosolubilizer ingredients of the lotion formulation to produce a highly favourable partitioning environment for the corticosteroid.

The peak blanching produced under occlusion by the scalp application and cream formulations each increase by approximately 15% compared to the unoccluded results. Again the additional moisture in the skin must improve partitioning or facilitate drug diffusion through the skin strata that have swollen as a result of the higher water content. It is possible that not only does the occlusion trap transpirational moisture in the skin, but that the

aqueous dosage forms may supply some of the water that is participating in the hydrating process. On the other hand, the increase in blanching observed for the ointment is a relatively small 3%. The inherently occlusive nature of the ointment may induce near-maximal hydration of the stratum corneum when this product is applied without further occlusive wrapping. The subsequent use of occlusive tape, as practised in the occluded assay mode, therefore adds little to the occlusion already afforded by the applied ointment and, hence, the relative increase in drug permeation observed in the occluded mode is minimal.

Statistically, the blanching induced by the scalp application is significantly superior to that of the ointment formulation between the 7- and 18-hour observation times, inclusive, when assessed by the graded response method, whereas significant differences are demonstrated at every observation time when the blanching at adjacent sites is compared. Similarly, by the graded response comparison, significant superiority is demonstrated for the scalp application over the blanching elicited by the cream at observation times between 7 and 18 hours, and at the 32-hour reading. The comparison of blanching at neighbouring sites yields significant differences between the scalp application and cream blanching profiles at every observation time. Finally, the blanching values for the scalp application are significantly greater than those of the lotion formulation between 7 and 16 hours by the graded response comparison, and between 7 and 18 hours by the adjacent site comparison. Hence, the drug release from the scalp application is statistically superior to that from every other formulation at all observation times up to the 18-hour period.

The blanching produced by the lotion formulation is significantly greater than that produced by the ointment at all observation times between 7 and 16 hours, as assessed by either the graded response or adjacent site statistical comparison techniques. The difference between the data of these two formulations is also significant at the 28-hour reading times when the blanching at neighbouring sites is compared. A comparison of the lotion and cream blanching values by the graded response method yields statistically significant differences at all observation times between 7 and 16 hours with the exception of the 9-hour reading. The comparison of blanching at adjacent sites demonstrates significant differences between the lotion and cream formulations at all observation times between 7 and 14 hours, and at the 28-hour reading.

Both statistical tests indicate that the degrees of blanching produced by the cream and ointment formulations are significantly different at all observation times between 7 and 12 hours, and, additionally, the graded response test indicates significant difference at the 16-hour observation time.

In summary, the hydration of the stratum corneum induced by the occlusive covering significantly influences the drug partitioning between applied product and skin, and the diffusion of the drug through the skin strata. This is especially apparent for the solution and aqueous dosage forms but does not seem instrumental in improving drug permeation from the ointment environment. The blanching profiles elicited by all four preparations in the occluded mode are similar in shape and in the time-to-peak values. In contrast to the unoccluded mode, the lotion and cream formulations demonstrate superior blanching to the ointment, while drug permeation from the scalp application is maximal in both modes. There are significant differences between all four profiles indicating statistically real superiority in the drug release potential of certain formulations over that of others. In every comparison the significant differences in the blanching produced are apparent at the first observation time (7 hours), and these disparities remain statistically significant until well after the period of maximum blanching. These blanching differences have been ascribed to drug partitioning factors and the augmentation of diffusion through the stratum corneum by partitioned formulation ingredients.

EXTEMPORANEOUS RESORCINOL CREAMS

Twelve volunteers were used to assess the blanching responses to three extemporaneous buffered cream formulations containing 0%, 2% or 5% resorcinol and commercial Betnovate cream. The extemporaneous creams were compounded to contain 0.1% betamethasone (as the 17-valerate) and the assayed percentage purities were 103.0%, 97.5% and 98.3% for the products containing 0%, 2% and 5% resorcinol, respectively. The label strength of the Betnovate cream was 0.1% betamethasone (as the 17-valerate) and assay of this preparation yielded a percentage purity of 95.04%. All the products were, therefore, within the pharmacopoeial limits for drug content. The commercial product was included in the trial simply as a reference standard against which the blanching elicited by the compounded creams could be compared. The blanching trial was conducted in both the unoccluded and occluded modes, each preparation being applied to three forearm sites of each volunteer in each application mode. The results of this assay are presented in Tables XLVI and XLVII, and plotted in Figures 51 and 52, respectively.

Immediately apparent from Figure 51 is the marked difference in the degree of blanching produced by the commercial Betnovate cream in comparison to that elicited by the extemporaneously manufactured products in the

TABLE XLVI: Blanching response to extemporaneous betamethasone 17-valerate buffered cream formulations containing 0%, 2% or 5% resorcinol and Betnovate cream applied in the unoccluded mode.(%TPS)

Time (hours)	Resorcinol content 0%	2%	5%	Betnovate cream
7	26.157	28.241	21.296	12.269
8	30.324	31.482	26.389	14.120
9	34.954	35.185	28.704	17.130
10	37.037	34.722	32.176	19.444
12	48.843	46.528	41.898	25.000
14	57.407	53.935	49.306	28.935
16	60.185	54.630	50.694	28.241
18	52.778	47.685	46.065	22.454
28	25.232	19.676	20.602	8.796
32	16.898	15.972	17.825	7.870
AUC	1085.417	997.685	928.588	485.880

TABLE XLVII: Blanching response to extemporaneous betamethasone 17-valerate buffered cream formulations containing 0%, 2% or 5% resorcinol and Betnovate cream applied in the occluded mode.(%TPS).

Time (hours)	Resorcinol content 0%	2%	5%	Betnovate cream
7	35.185	40.278	38.194	28.935
8	40.741	44.907	44.444	32.407
9	48.843	52.546	53.241	39.120
10	53.704	56.944	57.176	42.130
12	64.352	68.056	66.204	53.009
14	64.352	69.213	66.898	50.926
16	59.259	61.574	59.491	43.056
18	50.926	52.546	50.000	36.806
28	17.361	19.907	18.750	11.111
32	13.889	13.657	13.889	8.333
AUC	1141.667	1223.611	1180.440	859.722

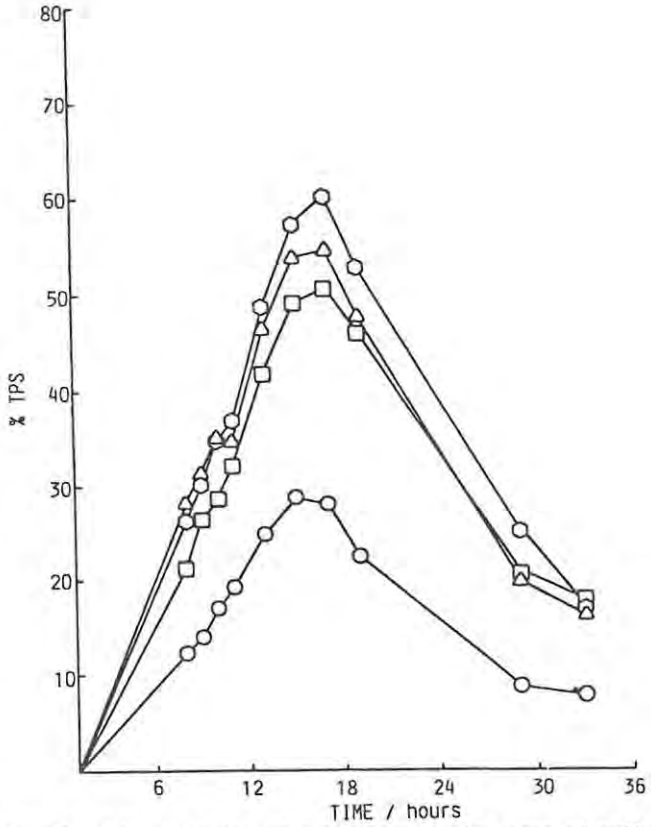


FIGURE 51: Blanching profiles of extemporaneously prepared buffered cream formulations containing various porportions of resorcinol, and Betnovate cream, assessed in the unoccluded application mode. ○ Betnovate, ⬡ 0% resorcinol, △ 2% resorcinol, □ 5% resorcinol.

unoccluded mode. It was assumed that the drug bioavailability from the formulated creams may approach that from the proprietary product, but a greater degree of drug release from these extemporaneous products was not anticipated. The buffered cream products are clearly superior to Betnovate in their ability to release betamethasone 17-valerate. The rank order of blanching for the buffered cream formulations appears to decrease with the increasing concentration of resorcinol in each product. This suggests that some form of corticosteroid-resorcinol interaction may be occurring within the cream vehicle which inhibits drug partitioning into the skin.

Statistically, there is significant difference in the blanching produced by the Betnovate product compared to the blanching elicited by all three of the buffered cream formulations at every observation time during the 7- to 32-hour reading interval. These significant differences are demonstrated for both the graded response and comparison of adjacent site statistical tests.

There is significant difference, by the graded response test, between the results of the 0%- and 2%-resorcinol creams at the 18-hour observation time. Additionally, the adjacent site comparison test applied to the results from these two creams demonstrates statistical significance at the 14-, 28- and 32-hour observation times.

The graded response test shows significant difference between the blanching of the 0%- and 5%-resorcinol creams at the 18-hour reading period, while the adjacent site comparison demonstrates significant differences between the results of the 2%- and 5%-resorcinol creams between 7 and 9 hours.

Therefore, the blanching elicited by all three buffered cream formulations is statistically superior to that of the Betnovate cream at all observation times. On the other hand, the results from the three extemporaneous formulations are essentially equivalent, with the possible exception of the 18- to 32-hour interval, even though a rank order difference between the results is apparent. At the 18-hour observation the blanching produced by the 0%-resorcinol cream is significantly superior to that of either the 2% or 5% resorcinol-containing creams, there is no statistical difference between the results of the latter two creams at this time.

The occluded application mode (Figure 52) similarly demonstrates the superiority in drug release potential of the buffered cream formulations in comparison to the commercial Betnovate cream. As before, the time-to-peak values have decreased by approximately two hours by the use of occlusion, and the peak %TPS values have also increased, markedly in some cases. The peak blanching value for the Betnovate cream has increased significantly from approximately 29%, in the unoccluded mode, to 51% with occlusion. Similarly the peak values of the 2% and 5% resorcinol-containing creams have increased by approximately 14% and 16%, respectively. In contrast, the peak value of the 0%-resorcinol cream only demonstrates a 4% increase under occlusion. The rank order increase in blanching elicited by the buffered cream products is in agreement with the concentration of resorcinol incorporated into the formulations. It is possible that the greater proportion of transpirational water present in the stratum corneum in the occluded mode is reacting in some fashion with the resorcinol of the vehicle to enhance drug partitioning or improve corticosteroid diffusion through the stratum corneum.

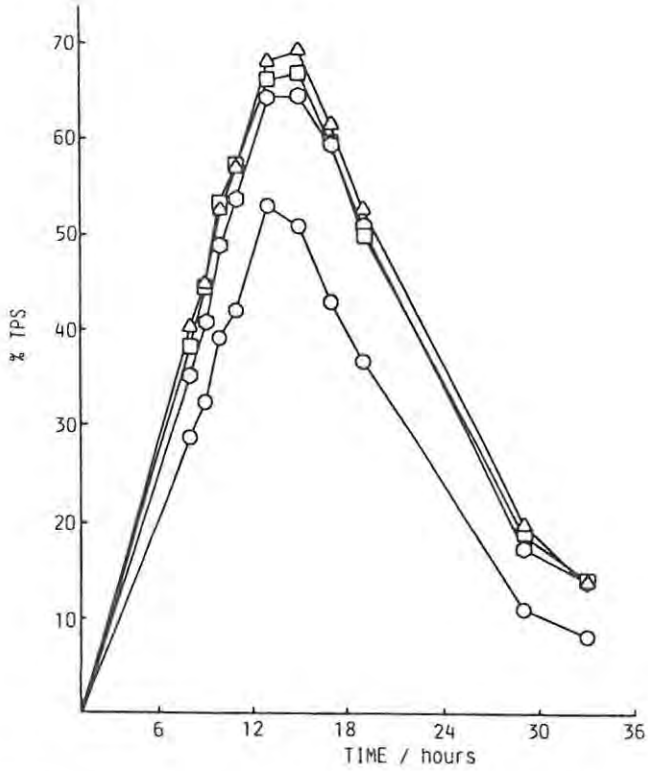


FIGURE 52: Blanching profiles of extemporaneously prepared buffered cream formulations containing various porportions of resorcinol, and Betnovate cream, assessed in the occluded application mode.
○ Betnovate, ○ 0% resorcinol, △ 2% resorcinol, □ 5% resorcinol.

This would account for the relatively greater increase in peak blanching observed for the resorcinol-containing creams compared to that of the 0%-resorcinol cream. Statistically, there is significant difference between the blanching produced by both the 2% or 5% resorcinol-containing creams and that produced by the Betnovate product at all observation times between 7 and 32 hours, as assessed by both the graded response and adjacent site comparison methods. There are significant graded response differences between the blanching of the 0%-resorcinol cream and that of Betnovate cream at 7 hours, between 9 and 18 hours, and at the 32-hour observation, whereas significant differences are demonstrated between the blanching of these two products at every observation time by the adjacent site comparison method.

Throughout the assay period the graded response statistical method demonstrates no significant differences between the blanching results of all three buffered cream formulations. On the other hand, the adjacent site comparison method demonstrates significant differences between the blanching results of the 0%-resorcinol cream and that of the 2% resorcinol-containing cream between 7 and 10 hours, and at the 14- and 28-hour observation times. Similarly, differences in blanching are significant between the 2% and 5% resorcinol-containing creams between 7 and 9 hours, between 12 and 18 hours, and at the 32-hour observation.

In summary, the blanching elicited by each of the buffered cream formulations is statistically greater than that produced by the commercial Betnovate cream in the occluded mode of application. Essentially the results from the three extemporaneously prepared products are equivalent, even though a rank order difference is apparent which suggests superiority in drug release of the 2%-resorcinol formulation over that of the 5%- or 0%-resorcinol products. In agreement,the results from the statistical method comparing blanching at adjacent sites suggests that the blanching of the 2% resorcinol-containing cream is significantly superior to that of either the 0% or 5% formulations, whereas the results from the latter two preparations are equivalent.

EXTEMPORANEOUS UREA CREAMS

Twelve volunteers were used to assess the blanching responses to extemporaneously prepared buffered cream formulations containing 0% or 10% urea and commercial Betnovate cream. The extemporaneous creams were compounded to contain 0.1% betamethasone (as the 17-valerate) and the assayed percentage purities were 103.0% and 97.6% for the products containing 0% and 10% urea, respectively. The label strength of the Betnovate cream was 0.1% betamethasone (as the 17-valerate) and assay of this preparation yielded a percentage purity of 95.04%. All the products were, therefore, within the pharmacopoeial limits for drug content. The commercial product was included in the trial as a reference standard against which the blanching elicited by the compounded creams could be compared. The blanching trial was conducted in both the unoccluded and occluded modes, each preparation being applied to four sites on the forearm of each volunteer in each application mode. The results of this assay are presented in Tables XLVIII and XLIX, and plotted in Figures 53 and 54, respectively.

TABLE XLVIII: Blanching response to extemporaneous buffered cream formulations containing 0% and 10% urea, and Betnovate cream applied in the unoccluded mode. (%TPS).

Time (hours)	Urea content 0%	Urea content 10%	Betnovate cream
7	24.306	18.287	16.204
8	30.324	22.917	20.139
9	39.583	27.778	24.306
10	47.454	37.732	33.796
12	58.565	46.065	42.130
14	66.204	51.389	43.982
16	60.880	45.139	34.491
18	56.019	39.120	26.852
28	18.750	17.130	6.482
32	9.491	9.259	4.861
AUC	1095.949	838.773	617.361

TABLE XLIX: Blanching response to extemporaneous buffered cream formulations containing 0% and 10% urea, and Betnovate cream applied in the occluded mode. (%TPS).

Time (hours)	Urea content 0%	Urea content 10%	Betnovate cream
7	35.880	35.648	34.259
8	43.519	45.370	40.741
9	52.778	54.398	51.157
10	59.954	62.500	58.333
12	67.824	70.370	67.361
14	66.435	68.519	61.806
16	55.324	55.093	48.380
18	44.444	45.602	39.120
28	13.889	13.436	11.574
32	6.481	5.093	6.250
AUC	1085.764	1101.852	999.769

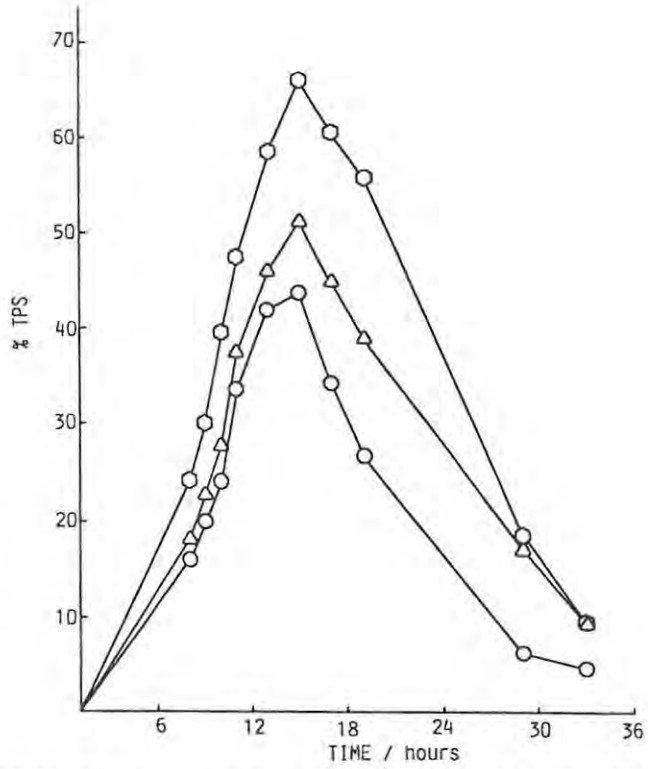


FIGURE 53: Blanching profiles of extemporaneously prepared buffered cream formulations containing various porportions of urea, and Betnovate cream, assessed in the unoccluded application mode.
○ Betnovate, ○ 0% urea, △ 10% urea.

Certain trends are common to the unoccluded results of this blanching assay and those from the previously reported trial using resorcinol-containing creams. In both cases the commercial Betnovate product induces a lesser degree of blanching than any of the extemporaneously prepared creams. In the unoccluded mode of both trials the buffered cream containing neither resorcinol nor urea elicits a greater degree of blanching than the same base containing these agents. Furthermore, in both trials the unoccluded blanching profiles are broader and blunter than the profiles from the occluded application mode, suggesting an apparently longer duration of corticosteroid action in the former mode.

It can be seen from Figure 53 that the 10% urea-containing cream produces a degree of blanching intermediate between that of the 0%-urea and the Betnovate formulations. The interpretation of this result may validate the theory proposed previously if it is assumed that interaction of the corticosteroid and urea molecules within the cream vehicle is creating a favourable environment for retention of the drug in the formulation, and thereby inhibiting drug partitioning into the skin. The nature of this possible interaction is unclear, the diamide structure of urea is thought to form a resonance hybrid with positive charge delocalization over the amino groups and a negative charge resident on the carbonyl oxygen. This charged species should interact

readily with other polar moieties (other urea molecules for example) by transient ionic bonding to form relatively large, loose complexes in the cream vehicle. It may be possible that these ionic aggregates assist in the cosolubilization of betamethasone 17-valerate or augment its stabilization in solution. It is certain that in the unoccluded assay mode the presence of urea in the buffered cream formulation decreases the affinity of the drug for the stratum corneum.

Statistically, there are significant graded response differences between the blanching values of Betnovate cream and those of the 0%-urea cream from the 8-hour to the 28-hour observation times. Additionally, comparison of blanching at adjacent sites also demonstrates significant differences at the 7-hour reading. The graded response values of the 0%-urea cream are significantly different to those of the 10% urea-containing formulation between 9 and 18 hours, and, in addition to these times, the comparison of adjacent site blanching demonstrates significant differences at the 8-hour observation. The graded response values of the 10% urea-containing cream are significantly different to those of the Betnovate product only in the 14-18 hour interval, whereas significant differences are demonstrated between the blanching of these two preparations at every observation time by the comparison of adjacent sites method.

In summary, the blanching profiles produced by the three preparations in the unoccluded mode are similar in shape and time-to-peak values. There are significant differences between the blanching produced by the 0%-urea cream and that elicited by both the 10%-urea formulation and Betnovate cream at all observation times except the first and last readings. The differences between the blanching elicited by the 10%-urea cream and that of the Betnovate product are more marked after 14 hours than in the interval prior to peak blanching, however, drug release superiority of the urea formulation is certain. The lesser degree of blanching generated by the urea-containing formulation, as compared to the 0%-urea base, has been ascribed to intraformulation interaction between corticosteroid and this adjuvent.

As before, occlusion of these applied products has generated blanching profiles that peak approximately two hours earlier than in the unoccluded mode, and the peak blanching values are greater for some of the formulations. Occlusion of the Betnovate cream has increased its peak value by approximately 23% compared to the unoccluded results, similarly the peak value of the urea formulation has increased by approximately 19%. In contrast, the peak blanching of the 0%-urea cream has only increased by 2% under occlusion.

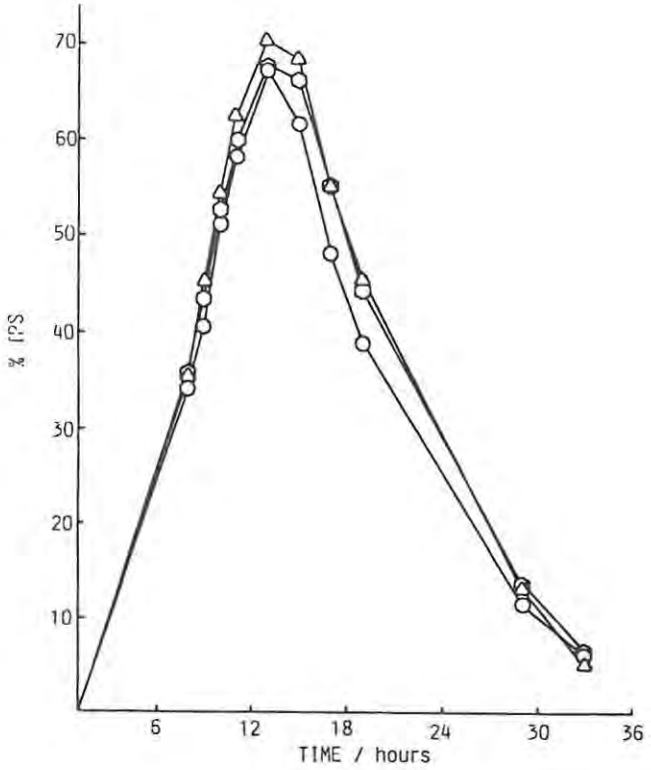


FIGURE 54: Blanching profiles of extemporaneously prepared buffered cream formulations containing various porportions of urea, and Betnovate cream, assessed in the occluded application mode.
○Betnovate, ○ 0% urea, △10% urea.

This result is in close agreement with that observed in the resorcinol trial and may suggest that the formulation constituents are interacting maximally with the available transpirational moisture of the stratum corneum, even in the unoccluded mode, to facilitate drug partitioning from the base. On the other hand, the greater proportion of water present in the occluded mode may interact with the polar urea molecules, enhancing their movement into the skin strata. The presence of urea in the horny layer and its translocation from the formulation may facilitate the carriage or partitioning of the betamethasone 17-valerate molecules, thereby eliciting the greater degree of blanching observed. It is certain that the polar urea moieties must interact in some fashion with the equally polar water molecules of the stratum corneum and this interaction augments drug release from the formulation.

Similarly, occlusion of the Betnovate cream markedly increases its drug release potential. One can only speculate on the interaction here as the exact composition of the proprietary formulation is unknown. Again, the water and certain of the Betnovate cream constituents must interact in such a manner that corticosteroid partitioning into the skin is favoured.

The three profiles obtained in the occluded mode (Figure 54) are very similar in both shape and magnitude, and, hence, there are relatively few instances of statistically significant differences. The graded response

blanching values of the 0%-urea base are significantly different to those of the Betnovate cream only at the 9-hour observation time. On the other hand, the comparison of blanching at adjacent sites demonstrates significant differences between these formulations at the 8-, 9- and 10-hour readings, and at all times during the 14- to 32-hour interval. Similarly, the graded response blanching values of the 10%-urea formulation are significantly different to those of the Betnovate product only at the 16-hour observation time, whereas there are significant differences between the blanching produced by these two formulations at 10 hours, and during the 14- to 18-hour interval by the comparison of blanching at adjacent sites test. Both the graded response and comparison of blanching at adjacent sites tests demonstrate no significant differences between the results of the 0%-urea cream and those of the base containing 10% urea at any observation time.

In summary, the profiles obtained in the occluded application mode for all three formulations peak at earlier times and are of greater magnitude than those observed in the unoccluded modes. The occluded data values are almost coincident at many observation times and, hence, there are few instances of statistically significant differences. These instances occur mainly after the period of peak blanching. Although the three profiles are, therefore, essentially equivalent, the rank order of blanching observed is the 10%-urea formulation greater than the 0%-urea cream, which in turn is greater than Betnovate cream. Superiority in drug release potential of the urea-containing formulation has been ascribed to polar interactions of the adjuvent with water molecules trapped within the stratum corneum, thereby augmenting corticosteroid partitioning and diffusion.

4.5 COMPARISON OF BLANCHING AND *IN VITRO* PERMEATION RESULTS

The objective in the development of this new *in vitro* diffusion cell was to establish a system that could easily and rapidly estimate the rate and extent of transdermal corticosteroid permeation from different topical formulations. The availability of such a system would greatly facilitate the testing of new drugs for potency or new topical vehicles for their drug release potential. Furthermore, this *in vitro* data may eliminate the need for conducting repetitive trials using human volunteers. In most cases the testing of new topical formulations would be conducted on a comparative basis,

drug permeation from the test formulation being compared to that from a characterized, standard product. If the results of these *in vitro* investigations show that a specific membrane adequately simulates the *in vivo* drug permeation of betamethasone 17-valerate from a particular class of topical dosage form (cream, lotion, ointment, solution), then it may be assumed that valid comparative results would be obtained by using this membrane/diffusion cell combination for testing other products in the same formulation class containing the same corticosteroid.

It is prudent that a comparison of the data presented in Sections 4.3 and 4.5 be made to assess the possible correlation of the *in vitro* laboratory results with those observed in the blanching trials, which, theoretically, mimic the conditions of clinical product usage. Direct quantitative comparison of the results is impossible as the blanching assay employs a subjective assessment method. Notwithstanding this limitation, a comparison of the rank order of drug permeation from the different products through the various membranes with the rank order of blanching elicited by these same formulations would indicate the instances of close *in vitro–in vivo* correlation. A summary of the rank order drug permeation and blanching results from Sections 4.3 and 4.4 is given in Table L. This summary is presented simply in terms of the response-time profile for drug release from one formulation being greater than or equal to that from another formulation. Where the permeation profile for one product is greater than, but similar to, that of another product the result is reported as $\geq$. In addition, the rank order statistical analyses are also given in parentheses.

TABLE L: Rank order summary of *in vitro* permeation data and *in vivo* blanching assay results for the commercial and extemporaneous betamethasone 17-valerate topical formulations.

Formulation	*In vitro* membrane used				Application mode *in vivo*	
	Silicone	Human[a]	Pig[a]	Mouse[b]	Occluded	Unoccluded
Commercial Creams	P>B=C (P=C=B)	P>C>B (P=C=B)	P>C>B (P=C≥B)	C=B=P (P=C=B)		P>B>C (P>B=C)
Commercial Lotions	C>B (C>B)	C=B (C=B)	C>B (C=B)	C=B (C=B)		C=B (C=B)
Commercial Ointments	C>B>P (C>B>P)	C=P>B (C=P>B)	P>C>B (P>C=B)	P>C>B (P=C>B)		P>C>B (P>C≥B)
Resorcinol Cream				5%>2%>0% (5%>2%>0%)	2%>5%>0%>B (2%≥5%=0%>B)	0%>2%>5%>B (0%≥2%≥5%>B)
Urea Cream				10%=0% (10%=0%)	10%>0%>B (10%=0%=B)	0>10%>B (0%>10%≥B)
Betnovate Products	S>O>L>CR (S>O>L≥CR)	S>L>O=CR (S>L>O=CR)	S>CR=L>O (S>CR=L≥O)	S>L>CR>O (S>L>CR>O)	S>L>CR>O (S>L>CR>O)	S>O>CR>L (S>O=CR>L)

Rank order results of statistical analysis in parentheses.
B Betnovate, C Celestoderm-V, CR Cream, L Lotion, O Ointment, P Persivate, S Scalp application. [a] Stratum corneum, [b] Full thickness skin.

CREAMS

The greatest degree of betamethasone 17-valerate permeation through silicone membrane, human and weanling pig stratum corneum is experienced from Persivate cream. This formulation also generates the greatest degree of blanching in the unoccluded application mode. Drug permeation from Persivate cream through hairless mouse skin appears lowest in rank order but this flux rate is statistically equivalent to that of either Betnovate or Celestoderm-V cream. This result may be explained by examination of the formulation assay values (Appendix 1) which demonstrates Persivate cream to contain approximately a 10%-greater concentration of betamethasone 17-valerate than either Betnovate or Celestoderm-V cream, which are both at full strength. The higher drug concentration is obviously providing a greater driving force for permeation in this case. However, there is generally no statistical difference between the drug permeation rates from the three formulations through any of the membranes studied, mainly due to the large standard deviations of the means experienced at each sampling time. On the other hand, the blanching results demonstrate significant statistical superiority of the Persivate formulation over Betnovate or Celestoderm-V, which are statistically equivalent.

Drug permeation from Celestoderm-V cream is greater than that from Betnovate through human and pig stratum corneum and hairless mouse skin, however the flux rates from these two formulations appear equal through silicone membrane and blanching is greater for the Betnovate formulation than for the Celestoderm-V cream in the unoccluded mode.

Close correlation is therefore demonstrated between the *in vitro* drug permeation results through human and weanling pig stratum corneum, and through silicone membrane, even though the permeation profile for the latter medium follows the square-root-of-time relationship. Furthermore, close agreement is demonstrated between the permeation results of all the membranes, except hairless mouse skin, and the unoccluded blanching data in ranking Persivate cream as the most capable of drug release. The permeation results from all the membranes, except weanling pig stratum corneum, indicate that there is little difference between the drug release rates from Betnovate and Celestoderm-V creams. These results show some agreement with the unoccluded blanching trial data which indicates a relatively small rank order superiority of Betnovate over Celestoderm-V. Similar blanching results have been reported by Meyer *(272)* and Coleman *(290)*.

Therefore, definite correlation of the *in vitro* permeation data with the unoccluded blanching assay results is evident. The closest rank order

agreement between the blanching and laboratory data is shown for the human stratum corneum, as expected, and silicone membrane. *In vitro-in vivo* agreement of data is observed to a lesser extent for weanling pig stratum corneum and hairless mouse skin. The correlation of the silicone membrane data is interesting in that it suggests a totally synthetic polymer medium may be used to compare the betamethasone 17-valerate permeation rates from cream formulations, a distinct advantage for *in vitro* research.

LOTIONS

The rank order of the *in vitro* betamethasone 17-valerate permeation through silicone membrane and weanling pig stratum corneum is greater from Celestoderm-V lotion than from Betnovate. This result would be expected as the assay values for the two formulations indicate that Celestoderm-V lotion, although at full strength, is approximately 10% more concentrated than Betnovate lotion. However, the greater degree of drug permeation from Celestoderm-V lotion is not exhibited through either human stratum corneum or full thickness hairless mouse skin and, furthermore, no significant differences in the *in vivo* unoccluded blanching profiles of the two products have been observed in this investigation or in that of Meyer *(272)*.

On the other hand, statistical significance in the difference between the *in vitro* permeation profiles from the two formulations is only demonstrated for the silicone data. The permeation data through all the other membranes and the *in vivo* blanching data shows no statistically significant difference between the drug release potentials of the two products.

Therefore, close correlation of the *in vitro* results with the unoccluded blanching assay data is demonstrated for the human stratum corneum and hairless mouse skin. The permeation data through both these membranes indicates that there is little difference in the corticosteroid release rates from the two lotion formulations, a result corroborated by the observed blanching. This indication is also given, to a lesser extent, by the permeation data through the weanling pig stratum corneum which demonstrates no significant statistical difference between the flux rates from the two lotions, but this data does suggest rank order superiority of the Celestoderm-V formulation.

The profiles of the silicone membrane and human stratum corneum both follow square-root-of-time relationships which may suggest that the two media function in a similar manner in retarding drug movement. It is therefore postulated that the difference in the Celestoderm-V and Betnovate profiles

observed through silicone membrane results from the drug concentration difference in the two formulations. It is possible that the profiles would have been more alike if the strengths of the lotions had been equal. The silicone medium obviously presents less of a barrier to corticosteroid movement than the stratum corneum, as evident by the flux magnitudes, and hence it is anticipated that a 10% concentration difference of the donor vehicles would manifest itself to a greater extent with the polymeric barrier.

It appears, therefore, that the closest correlation of the lotion permeation results with the unoccluded blanching assay data is obtained when human stratum corneum or hairless mouse skin are used *in vitro*. Statistical correlation may also be obtained using weanling pig stratum corneum however the rank order of permeation obtained with this membrane may be misleading. It is proposed that silicone membrane is better able to demonstrate a differential driving force for permeation, as generated by different drug concentration gradients, than it is able to mimic the barrier potential of the human stratum corneum.

OINTMENTS

The rank order *in vitro* permeation of betamethasone 17-valerate from the ointment products is similar through the weanling pig stratum corneum and hairless mouse skin. Both these membranes demonstrate drug release to be greatest from the Persivate formulation, of lesser magnitude for the Celestoderm-V ointment, and slowest from the Betnovate product. Statistically, drug release from Persivate is significantly greater than that from Celestoderm-V through the porcine medium but the flux rates from these products are not significantly different through the mouse skin. On the other hand, the drug release rate is significantly greater from Celestoderm-V than from Betnovate through the murine medium but the flux difference is not significant through pig stratum corneum.

This rank order agrees with the unoccluded results of the blanching trial which show that Persivate induces the most intense blanching, Celestoderm-V has an intermediate blanching profile, and Betnovate elicits the lowest degree of blanching. Statistically, the blanching trial results are significantly different for Persivate and Celestoderm-V whereas there are periods of no significant difference between the Celestoderm-V and Betnovate data. This rank order of blanching agrees with the results of Meyer *(272)* and Coleman *(290)* who note that there is little difference in the degree of blanching

produced by Celestoderm-V or Betnovate in either the occluded or unoccluded modes.

Drug permeation through the human stratum corneum is not significantly different from the Persivate and Celestoderm-V ointments but the flux rates from these formulations are significantly greater than that from Betnovate. Hence the *in vitro* human data does not agree that closely with the unoccluded blanching results inasmuch as there is no *in vitro* distinction between the flux rates from Persivate and Celestoderm-V. However, agreement between these data is apparent in demonstrating drug release to be slowest from Betnovate ointment.

These results are interesting from a formulation point of view if one examines the assay values for the creams. Celestoderm-V ointment has an assay value of approximately 88%, Betnovate is approximately 5% overstrength and Persivate ointment is full strength. Notwithstanding these concentrations, drug partitioning and permeation is lowest from the Betnovate product and relatively higher from the understrength Celestoderm-V ointment, which demonstrated a definite proportion of the 21-valerate degradation product in the assay chromatograms. No degradation product was quantified in the receptor chamber isopropyl myristate solution when the permeation from Celestoderm-V was tested which suggests that the blanching monitored in the *in vivo* assay was elicited mainly by the parent 17-valerate. Corticosteroid partitioning must, therefore, be more favourable from the Celestoderm-V formulation than from the Betnovate formulation to generate higher flux profiles of this nature, assuming that the drug molecules, once partitioned from the different formulations, would diffuse at similar rates through the same medium. The highly important role that the formulation plays in drug permeation is exemplified by these observations.

In contrast to the *in vitro* results of the animal media, drug permeation through silicone membrane is slowest from Persivate ointment, however rank order agreement is demonstrated for the flux rates from Celestoderm-V and Betnovate ointments. Statistically, drug permeation is significantly superior from Celestoderm-V than from Betnovate, which in turn has a significantly superior drug release rate to that of Persivate. Furthermore, drug permeation through this membrane follows the square-root-of-time relationship which is not exhibited by any of the other media.

Therefore, definite correlation is observed between the *in vivo*, unoccluded blanching trial data and *in vitro* drug permeation through either weanling pig stratum corneum or hairless mouse skin. It is proposed that these membranes would adequately mimic the unoccluded blanching mode in the

comparative testing of ointment formulations. The human *in vitro* data does not agree that closely with the blanching assay results. This is unexpected bearing in mind that essentially the same barrier to permeation is being tested in both cases, and, furthermore, the results from the cream and lotion comparisons indicated close agreement between the human *in vitro* data and the blanching results. At face value these observations suggest that, for ointments, the *in vitro* diffusion system using human stratum corneum does not adequately mimic the unoccluded blanching mode *in vivo*. Agreement of the results is even less apparent when one considers the silicone membrane. The rank order reversal of the permeation through this polymer, especially at the early sampling times, suggests that unique interactions may be occurring between the lipophilic formulation constituents and silicone environment which modify corticosteroid partitioning and diffusion through the medium.

EXTEMPORANEOUS RESORCINOL CREAMS

The mass of betamethasone 17-valerate permeating hairless mouse skin from the extemporaneously prepared buffered cream formulation containing 5% resorcinol is significantly superior to that permeating from the 2%-resorcinol formulation and to that from the product containing no resorcinol. Furthermore, the drug flux rate from the 2%-resorcinol product is significantly higher than that from the 0%-resorcinol formulation. The presence of resorcinol in the formulation, thus, influences drug partitioning and diffusion from this particular cream preparation. The assay values for all three products are similar indicating that the influences of different donor drug concentrations on the permeation or blanching profiles are minimal.

The rank order of blanching observed in the unoccluded mode is greatest for the formulation containing no resorcinol, slightly lower for the 2% and 5% resorcinol-containing formulations, in that order, and the least blanching was induced by the commercial Betnovate cream. However, statistically there are instances of no significant difference between the blanching profiles of the 0%, 2% and 5% resorcinol-containing creams, whereas all three extemporaneous creams produce significantly superior blanching to that of the Betnovate formulation.

The rank order of blanching is reversed in the occluded assay mode which demonstrates drug release to be greatest from the 2% resorcinol-containing cream, and slightly lower from the 5%-resorcinol and 0%-resorcinol formulations, in that order. However, these three profiles are similar in magnitude and no distinct superiority of one formulation over another is apparent.

In agreement with the unoccluded data, Betnovate cream elicits the smallest degree of blanching in the occluded mode. Statistically, the blanching profiles of the 0%- and 5%-resorcinol creams are not significantly different. There are instances of significant difference between the blanching elicited by the 2%- and 5%-resorcinol formulations and the blanching produced by all the extemporaneous formulations is significantly superior to that of Betnovate cream.

A distinct rank order reversal is apparent in the results of the *in vivo* blanching assay when the test formulations are applied under occlusive wrapping. A comparison of these data would suggest that permeation monitored by the *in vitro* diffusion cell system, using hairless mouse skin as the barrier, more closely simulates the occluded blanching mode than the unoccluded situation. This theory is not incongruous with the *in vitro* experimental protocol in which an infinite dose of the topical formulation is applied to the donor surface of the membrane. This thick layer of aqueous formulation may provide the moisture to hydrate the stratum corneum of the membrane and maintain it in a hydrated state for the duration of the experiment. This postulation would support the argument proposed in Section 4.4 in which it was suggested that the resorcinol and moisture present in the stratum corneum interact in some fashion to enhance corticosteroid partitioning and diffusion through the skin. If the application of an infinite dose of formulation does hydrate the horny layer and increase the proportion of moisture present at the membrane-formulation interface, and if the interaction of water and resorcinol does increase the movement of betamethasone 17-valerate into the skin, then greater flux rates would be expected from formulations containing larger percentages of resorcinol--these results have been observed using this *in vitro* methodology.

Therefore, the closest correlation of the *in vitro* drug permeation results, from the extemporaneously prepared creams through hairless mouse skin, with the *in vivo* blanching results is observed when the occluded application mode is employed.

EXTEMPORANEOUS UREA CREAMS

The *in vitro* drug permeation from the extemporaneous 10% urea-containing formulation was not significantly different to that from the formulation containing no urea throughout the 192-hour experiment. The mean permeation values were slightly higher for the urea formulation at the early sampling times but coincidence of the profiles was observed after 72 hours.

extemporaneous formulations, it is suggested that *in vitro* drug permeation from the ointment formulations through human stratum corneum may possibly simulate the occluded blanching mode more closely than the unoccluded mode. This would certainly explain the observed nonagreement of the results for the human tissue, an agreement that is expected because essentially the same barrier is being tested *in vitro* and *in vivo*.

BETNOVATE FORMULATIONS

The most interesting results, from a comparative point of view, are those of the four different topical Betnovate formulations. These products were tested by the application of equal masses of betamethasone 17-valerate, in different formulation volumes, to the forearm sites of the volunteers. Differences in the observed blanching were, therefore, due to the release potential of each formulation, as the donor drug concentration was standard throughout.

The unoccluded results of this assay show that the scalp application induces the greatest degree of blanching, followed, in rank order, by the ointment, cream and lotion dosage forms. Statistically the results of the ointment and cream formulations are not significantly different, whereas the blanching induced by the scalp application is significantly superior to that of the ointment, and the blanching elicited by the cream is significantly superior to that of the lotion.

Rank order reversal in the degree of blanching is observed in the occluded assay mode. While the scalp application, again, produces the greatest degree of blanching, it is followed in rank order by the lotion, cream and ointment dosage forms. Furthermore, the differences between all four permeation profiles are statistically significant. There are, therefore, real differences in the betamethasone 17-valerate release rates from these four dosage forms when tested in the occluded blanching mode. Reasons for these differences have been suggested in Section 4.4.

Comparison of these *in vivo* observations with *in vitro* data requires abstraction of the Betnovate permeation results from the tables presented in Section 4.3. Table LI summarizes the mean corticosteroid masses that permeated human stratum corneum *in vitro* from the different Betnovate topical dosage forms. This data is represented graphically in Figure 55 and depicts the markedly different rates at which the corticosteroid permeates human stratum corneum from the different formulations. Drug permeation is greatest from the scalp application, although the magnitude of the permeation from the

In the unoccluded mode the rank order of blanching is greatest for the formulation containing no urea, less blanching is generated by the 10% urea-containing formulation, and the least blanching is elicited by the commercial Betnovate cream. Statistically the difference between the 0%- and 10%-urea formulation profiles are significantly different, whereas there are instances of no significant difference between the profiles of the 10%-urea formulation and the Betnovate product. The assay values are similar for the preparations indicating that there is little difference in the concentration driving force causing the permeation. The differences in the degrees of observed blanching must, therefore, be caused by the formulation composition in each case.

In the occluded mode the rank order of blanching is greatest for the 10%-urea formulation, less blanching is generated by the 0%-urea product and the least blanching is elicited by Betnovate cream. However, the magnitudes of these three profiles are of the same order, the AUC values ranging from 1000 units for the Betnovate cream to 1086 units for the urea-containing formulation. Furthermore, there is no significant statistical difference between any two of the profiles. These observations suggest that there is essentially no difference between the drug release rates from the three formulations in the occluded blanching mode.

In both the *in vitro* and *in vivo* investigations the observed drug permeation is equivalent from the extemporaneous formulations, irrespective of their urea content. The results here are very similar to those observed with the resorcinol-containing extemporaneous formulations in that the *in vitro* permeation data correlates closely with the results of the occluded mode of the blanching assay. On the other hand, the unoccluded blanching assay results indicate that there are significantly different flux rates from the two products, contradicting the findings of the *in vitro* investigation. In explanation, the theory proposed for the resorcinol formulations appears highly feasible in that the application of an infinite dose of cream on to the donor surface of the stratum corneum *in vitro* hydrates this medium and maintains it in the hydrated state. Corticosteroid partitioning into and diffusion through these hydrated strata would certainly simulate the occluded application environment *in vivo* more closely than it would the unoccluded mode.

These observations may also help to explain the anomolous results obtained for drug permeation *in vitro* from the commercial ointments when human stratum corneum was used as the diffusion medium. In this case it was reported that the *in vitro* results did not correlate that closely with the unoccluded blanching results. In view of the observations made with the

TABLE LI: Mean drug mass permeating human stratum corneum from Betnovate formulations at 35 °C and 600 rpm. ($\mu g\ cm^{-2}$).

Time (hours)	Betnovate preparation			
	Cream	Lotion	Ointment	Scalp appl.
6				2.959
12-16		4.208		6.530
18-24	1.379	6.894	0.915	10.398
30-36		12.367		14.765
48	2.294		1.847	35.872
60		18.400		56.633
72	3.438		2.936	97.833
85		22.969		159.184
96-109	4.366	26.727	4.091	212.359
120-132	5.104	30.663	5.162	
144-156	6.691	33.586	6.450	
168-180	7.285	37.341	7.724	
192-200	8.125	40.800	9.283	

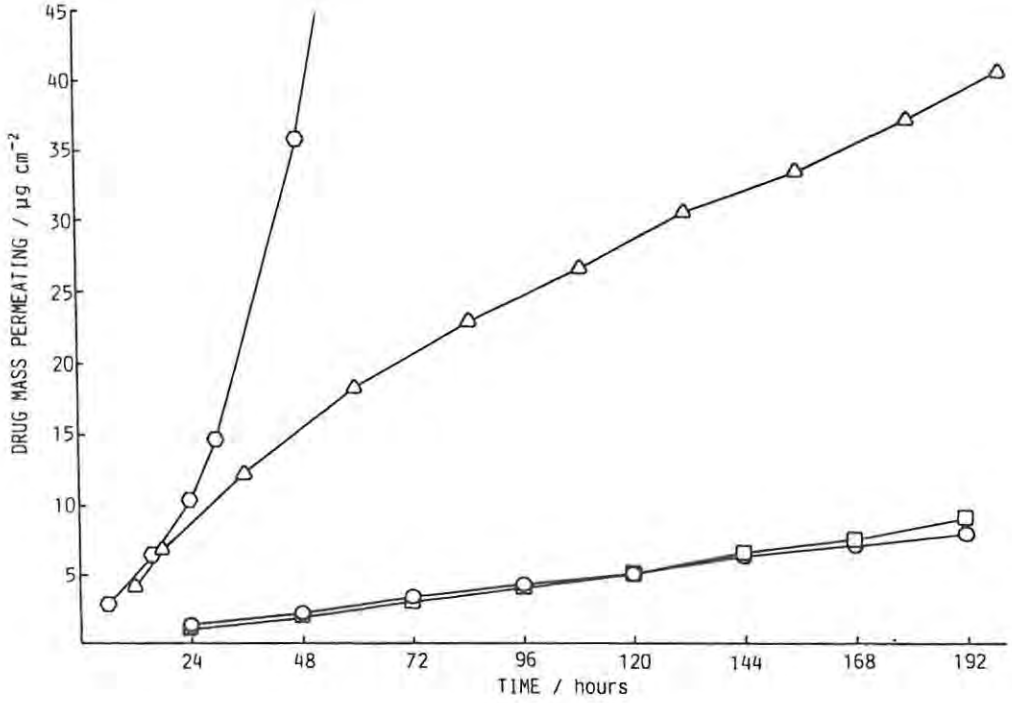


FIGURE 55: Permeation of betamethasone 17-valerate through human stratum corneum from Betnovate topical formulations at 600 rpm agitation and 35 °C. ○ cream, △ lotion, □ ointment, ○ scalp application.

lotion approaches that from the scalp application at early sampling times (16–18 hours). The differences between the scalp application and lotion profiles at this interval are not statistically significant. Thereafter marked deviation in these flux profiles is observed. On the other hand, the flux profiles for the cream and ointment dosage forms are similar at all sampling times, the mean permeation values being slightly greater for the cream than for the ointment at the majority of these periods. There is no statistical significance in the difference between these permeation rates.

It is therefore apparent that the *in vitro* permeation results, again, correlate closely with the occluded results from the *in vivo* blanching assay. In agreement with the comparisons reported above, the *in vitro–in vivo* correlation is not as obvious for the unoccluded blanching data. These observations certainly add credence to the *in vitro* occlusion theory postulated previously. In this regard it is interesting to note that the greatest proportional increase in blanching between the unoccluded and occluded modes is represented by the lotion data, especially as this formulation was assayed to be understrength (approximately 92%). This dosage form, being the least viscous of the emulgent formulations, is thought to contain the greatest proportion of water. It is suggested that this relatively large water fraction interacts with the stratum corneum, both in the occluded blanching mode and *in vitro*, substantially hydrating this layer and thereby facilitating drug partitioning and diffusion.

A relatively large increase in blanching under occlusion compared to the unoccluded mode is also observed for the full strength cream formulation, presumably for similar reasoning. It appears, therefore, that the cream and lotion dosage forms are supplying some of the water that is hydrating the horny layer under the occlusive dressings, whereas a large percentage of this moisture is probably lost to the atmosphere in the unoccluded mode. In addition, adjuvents, such as the glycols, may diffuse from these formulations and may assist in the solvation of the stratum corneum.

In contrast, the relative increase in the blanching generated by the 4%-overstrength ointment formulation under occlusion compared to the unoccluded mode is relatively small. It is known that this lipophilic formulation is inherently occlusive in nature and results in a certain degree of hydration of the stratum corneum *in vivo*. However, this hydration is generated by the prevention of endogenous moisture loss from the skin; there is no water provided for the hydrating process by the ointment itself. Hence, near-maximal transpirational hydration of the stratum corneum is generated, even in the unoccluded application mode. The subsequent occlusion of this dosage form with impervious wrapping, therefore, adds little to the hydrating process and an insignificant increase in the degree of blanching is observed. These observations have interesting clinical implications in that they suggest there will be greater betamethasone 17-valerate permeation from lotions or creams that are applied to dermatoses under occlusion than from ointments applied under similar wrapping.

On the other hand, the observed drug permeation is greatest from the scalp application at all observation times by both *in vitro* and *in vivo* monitoring methods. While the high assay value for the scalp application

(122%) must enhance the rate of drug permeation *in vitro*, it is believed that the alcoholic component in this dosage form plays a major role in either improving the partitioning environment into the skin or reducing the barrier potential of the stratum corneum, especially after prolonged contact, or a combination of these mechanisms may exist.

Therefore, from the close correlation of the results, it appears that the *in vitro* methodology using human stratum corneum mimics the occluded assay mode of the blanching trials (for betamethasone 17-valerate as the permeating species). Similar abstraction of the Betnovate *in vitro* permeation results from Section 4.3 using the other animal and synthetic membranes allows comparison of these data with that from the human blanching trial. These comparisons are summarized in Table L and indicate that the greatest degree of *in vitro* permeation is obtained from the scalp application through each membrane tested. In all cases the rank superiority of the scalp application permeation profile over those of the other dosage forms is statistically significant.

For the silicone membrane the next greatest permeation magnitude after the scalp application is obtained for the ointment formulation, which, in turn, is significantly greater than the results for the lotion. Drug permeation is slowest through the silicone medium from the cream dosage form, however there are instances where the differences between the cream and lotion results are not statistically significant. This rank order of *in vitro* permeation, therefore, appears to correlate more closely with the results of the unoccluded blanching assay which also demonstrates drug release from the ointment formulation to be second in rank order. This does not detract from the validity of the *in vitro* occlusion theory proposed above in that it is not anticipated that the topical formulations would modify the polymeric environment to any significant degree. The extent of hydration achieved by the animal media would certainly not be approached with this synthetic membrane. Hence, it is anticipated that the silicone results would resemble those from a nonhydrated biological membrane, as demonstrated by the unoccluded blanching data. However, this correlation of the *in vitro* silicone membrane data with the unoccluded blanching trial results does not suggest correlation will be achieved between these assessment modes in every case. Re-examination of the results from the *in vitro-in vivo* comparisons for the commercial lotions and ointments demonstrates little agreement of the silicone rank order permeation with the unoccluded blanching assay results obtained for these dosage forms.

On the other hand, it may be proposed that the silicone membrane is adequately able to distinguish between drug release rates from the different

formulation types, in contrast to distinguishing between the release rates from different products of the same formulation type, and that this rank order distinction simulates that observed by the unoccluded mode of the blanching assay.

The rank order of *in vitro* permeation through the weanling pig stratum corneum is greatest from the scalp application, essentially equal from the cream and lotion dosage forms, and the least permeation is experienced from the ointment formulation. The statistical analysis of these profiles demonstrates a similar significance pattern: the results of the scalp application are significantly superior to those of both the cream and the lotion, which are not significantly different to one another, and the cream and lotion results are significantly superior to those of the ointment only at certain sampling times.

Hence, fair correlation is also demonstrated between the *in vitro* results using porcine membrane and the occluded *in vivo* blanching results. A definite statistical distinction between the permeation rates from the cream, lotion and ointment formulations is impaired by the large standard deviations of the means observed at each sampling time, which tend to decrease the significance of the difference between the average values. As stated previously, improved membrane excision and preparative techniques would concomitantly improve the uniformity of the medium and smaller variance would therefore be expected in the results. It is anticipated that a clearer statistical distinction between the permeation rates from these formulations would also be observed as a result.

By far the closest correlation of the *in vitro* results with the occluded blanching assay data is demonstrated for the hairless mouse skin. This membrane clearly shows the drug permeation rate from the scalp application to be significantly superior to that from the lotion, which, in turn, is significantly superior to that from the cream. Conversely, the drug permeation rate from the ointment is significantly lower than that from the cream. The same rank order and statistical evaluation is obtained from the occluded results of the blanching trial. The hairless mouse skin, therefore, appears able to distinguish between the drug release rates from the different formulation types exceptionally well, and this distinction correlates closely with the ability of the occluded mode of the blanching assay to distinguish between the same release rates. Retrospective examination of the comparative results for the extemporaneous cream formulations presented above indicates that the hairless mouse skin also makes adequate distinction between the drug release rates from similar products within the same formulation group. In

contrast, comparison of the results from the commercial creams indicates that hairless mouse skin was less able to distinguish between the drug permeation rates from the three proprietary formulations than human stratum corneum.

In summary, it is proposed that human stratum corneum is the most appropriate membrane to use for *in vitro* permeation studies. The results of these investigations have shown close correlation of the human *in vitro* data with *in vivo* data for the commercial creams and lotions, the Betnovate dosage forms, and, to a lesser extent, for the commercial ointments. Good correlation of *in vitro* and *in vivo* data has been demonstrated for the hairless mouse skin when used to monitor drug permeation from lotions, ointments and the Betnovate formulations. Correlation has been observed between the weanling pig stratum corneum results and blanching data for the commercial ointments, and to a lesser degree for the creams, lotions and Betnovate dosage forms, however these results may be improved upon by optimizing membrane preparation. The synthetic silicone medium appears to have very selective usefulness in this *in vitro* permeation methodology. While this medium is able to distinguish between the drug release rates from different classes of dosage form, it appears less able to simulate the *in vivo* permeation from different products of the same formulation class. It must be borne in mind that these correlations may only be applicable to the diffusion cell system used in this investigation to measure betamethasone 17-valerate permeation through membranes prepared by the techniques described above. Alternate cell designs or preparative techniques may produce dissimilar permeation results to those reported here.

Although good *in vitro-in vivo* correlation has been demonstrated for the diffusion cell results with the occluded blanching assay data, it is proposed that the results may agree more closely if similar experimental protocols are adopted for both assays. One facet of the methodologies that differs markedly is the drug permeation monitoring time. In the *in vivo* assay the peak blanching is observed at approximately 12-14 hours, after which a decline in the response is noted. In contrast, the *in vitro* protocol has monitored continuously increasing permeation for 200 hours. It is proposed that if the early *in vitro* permeation interval (up to 24 hours) could be characterized more fully then closer agreement of the resultant data with that of the occluded blanching assay would be observed.

The limits of detection of the high-performance liquid chromatograph (approximately 1 $\mu g\ cm^{-2}$) have usually dictated the first sampling time in the laboratory diffusion experiments. Although this has meant that samples

withdrawn from the receptor chamber at approximately 12 hours could be quantified for the lotion and scalp application donor formulations, the first quantifiable samples for the cream and ointment products were those withdrawn at approximately 24 hours.

It is possible, however, to compare the rank order drug permeation values at these early sampling times (12-14) hours, corresponding to the period of maximal blanching, with the rank order *in vivo* data during the same observation interval. The measured or estimated (by least squares regression analysis) *in vitro* drug permeation values for the Betnovate scalp application, lotion and cream formulations at the 12-hour sampling time are 5.26, 4.21 and 0.898 μg cm^{-2}. The estimated permeation value for the Betnovate ointment formulation at 14 hours is 0.336 μg cm^{-2}. These times correspond to the peak blanching periods in the occluded assay mode for each formulation. It is obvious from these results that close correlation exists between the rank order of permeation *in vitro* and the rank order of blanching *in vivo*, even at these relatively early *in vitro* sampling times. This observation is important in that it suggests that an *in vitro* experimental time of 16 hours, for example, would be sufficient to distinguish between the drug permeation rates from different formulations. Permeation runs lasting several days, as have been employed in this investigation, would, therefore, be unnecessary for comparative formulation testing. Furthermore, the possible degradative effects of prolonged exposure of the biological membranes to the experimental conditions would be avoided. However, these relatively short permeation experiments would require a diffusion cell system sensitive enough to fully characterize this early permeation phase.

Furthermore, there are several facets of the *in vivo* methodology that cannot be replicated exactly *in vitro*. A perpetual clearance of the drug by the dermal vasculature is experienced in the blanching assay, maintaining sink conditions, which is simulated by a large receptor volume *in vitro*. Metabolism and reservoir formation are extensive *in vivo*, and the stratum corneum cells are constantly desquamated from the exposed skin *(50)*; factors that may not be replicated by *in vitro* methodology.

5. PRELIMINARY TESTING OF HYDROCORTISONE AS A DIFFUSING SPECIES

It would obviously be beneficial if this new cell design could be employed to monitor the transmembrane permeation of corticosteroids other than betamethasone 17-valerate, or other classes of drugs entirely. To assess the capabilities of the new cell in this field a preliminary investigation was conducted to monitor the diffusion of hydrocortisone from two cream formulations, and to compare these *in vitro* flux data with results from an *in vivo* blanching trial.

The *in vivo*-mimic methodology was used to test the *in vitro* permeation of hydrocortisone from two formulations, proprietary Cutaderm cream (Scherag, South Africa) and an experimental cream formulation (Lennon, South Africa), each containing the drug at a concentration of 0.5%. Permeation was monitored at 35 °C from infinite doses of these formulations through full thickness hairless mouse skin, to a receptor phase of purified isopropyl myristate with chamber agitation of 600 rpm. Only one membrane was used in this preliminary investigation as the objective was simply to assess the feasibility of using the cell system to monitor the permeation of hydrocortisone, instead of betamethasone 17-valerate for which the cell had been validated. The *in vitro* drug permeation data from this investigation is presented in Table LII and plotted in Figure 56.

TABLE LII: Mean drug mass permeating hairless mouse skin from commercial hydrocortisone formulations at 35 °C and 600 rpm. ($\mu g\ cm^{-2}$).

Time (hours)	Formulation	
	Cutaderm	Lennon
6	0.588 (0.385)	0.653 (0.492)
12	1.524 (0.386)	1.468 (0.669)
24	3.398 (0.441)	3.152 (1.104)
31	4.936 (0.663)	5.165 (1.702)
48	8.854 (0.349)	9.102 (3.288)
72	16.916 (1.480)	16.144 (5.609)

Standard deviations of means in parentheses.

TABLE LIII: Blanching response to commercial hydrocortisone creams applied in the occluded mode. (%TPS)

Time (hours)	Formulation	
	Cutaderm	Lennon
7	17.130	20.833
8	30.556	28.935
9	33.102	33.796
10	37.731	41.435
12	44.213	46.065
14	38.426	43.519
16	24.463	29.167
18	16.898	22.222
28	8.102	10.185
AUC	546.750	629.800

The rate of hydrocortisone permeation through full thickness hairless mouse skin is, essentially, equal from both cream formulations. As can be seen from Figure 56 the two profiles intersect at various times and, statistically, the mean data values are not significantly different at any sampling time. For both creams the flux profile appears linear, especially during the first 48 hours, with a slight increase in the hydrocortisone permeation rate observed between 48 and 72 hours.

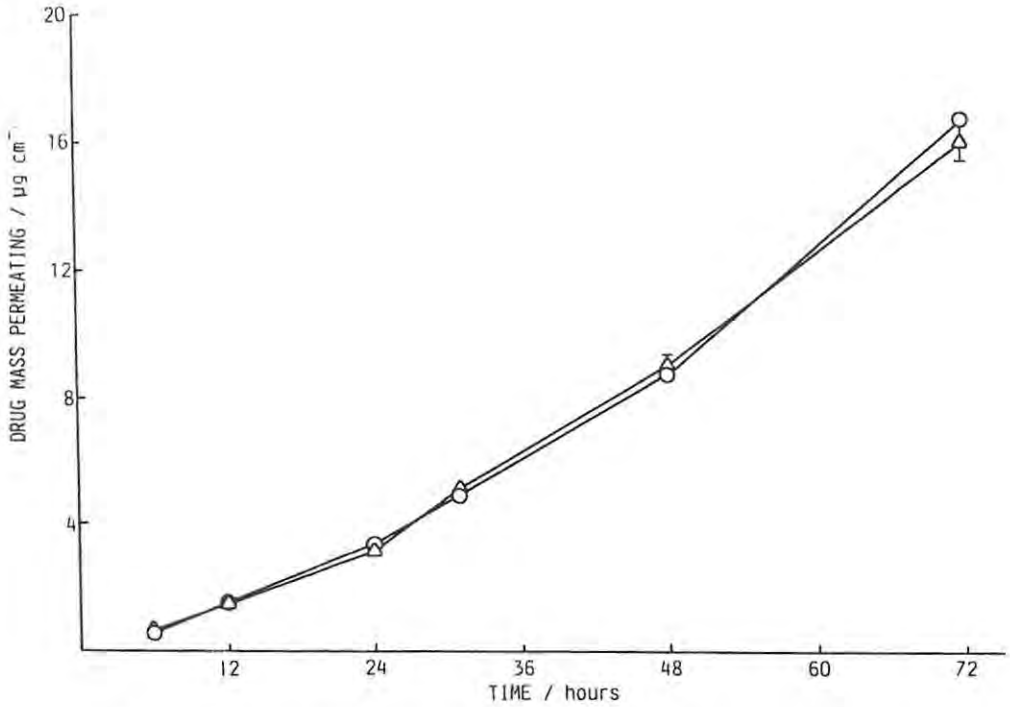


FIGURE 56: Permeation of hydrocortisone through full thickness hairless mouse skin from commercial cream, at 600 rpm agitation and 35 °C. ○ Cutaderm, △ Lennon.

From this data it may be concluded that there is no difference in the rate of drug release or diffusion through hairless mouse skin from Cutaderm cream and the experimental 0.5% hydrocortisone formulation. The greater sensitivity of the high-performance liquid chromatographic technique when assaying hydrocortisone allows receptor chamber permeant masses as low as 0.5 µg cm^{-2} to be quantified, in comparison to the 1.0-1.5 µg cm^{-2} range which marks the limit of detection for betamethasone 17-valerate. It is therefore possible to monitor hydrocortisone permeation with this cell system during the initial period of experimentation, the first quantifiable sample being withdrawn at six hours. In contrast, monitoring betamethasone 17-valerate permeation during the first 24 hours of experimentation was unreliable in most cases as the limit of detection was only attained towards the end of this initial period.

Twelve volunteers were used to assess the blanching responses to the two hydrocortisone creams, each preparation being applied to six sites on the forearm of each volunteer. Only the occluded application mode was used here as this relatively weak corticosteroid induces only slight blanching when tested without occlusion, which makes observation and interpretation of the results more difficult. For the same reason the maximum possible score used by the observers in assessing the graded blanching response was 2, instead of a maximum score of 4 used in the more potent betamethasone 17-valerate trials.

This modification allowed midrange %TPS values to be attained by both preparations, instead of very low %TPS values as would have resulted if the 0-4 graded response range had been used in the observations. The results of this blanching assay are presented in Table LIII and plotted in Figure 57.

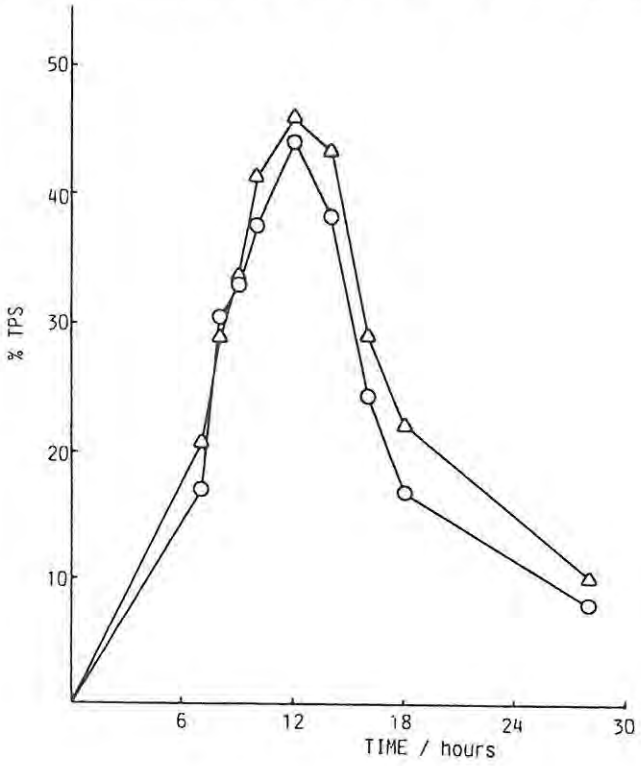


FIGURE 57: Blanching profiles of commercial and experimental hydrocortisone cream formulations assessed in the occluded application mode. ○ Cutaderm, △ Lennon.

These profiles indicate that the blanching elicited by the experimental Lennon formulation is greater in rank order than that of the Cutaderm product. However, the profiles are similarly shaped and both peak at approximately 12 hours. These profiles do not appear to be significantly different, except possibly towards the end of the observation time. This proposition is confirmed by statistical analysis which only yields one reading period, 18 hours, where the blanching data from the two creams is significantly different. Statistically, therefore, the degrees of blanching induced by the two formulations are not significantly different over the majority of the observation times.

Direct correlation of the *in vitro* permeation data with the *in vivo* blanching results is thus demonstrated in this investigation. The diffusion cell permeation data monitored over 72 hours indicates no significant differences in the drug release rates of the two hydrocortisone formulations, and, in congruity, the *in vivo* blanching data monitored over 28 hours

indicates an identical relationship in the degrees of blanching elicited by the two products. It appears, therefore, that the diffusion cell system is equally capable of monitoring the permeation of both betamethasone 17-valerate and hydrocortisone, and, moreover, sensitive characterization of the initial permeation period is possible with hydrocortisone. These data suggest that this cell system may be applicable in the monitoring of transdermal permeation of diverse steroidal classes, and possibly other drug families.

These *in vitro* results only serve as a preliminary investigation into the performance of the cell using hydrocortisone as the diffusing species, definite conclusions concerning the performance of the diffusion system can only be made after extensive investigations, as conducted for betamethasone 17-valerate, have been undertaken. Nevertheless, it is reassuring to note the close correlation of these data with that observed *in vivo* using the proven blanching assay.

6. IMPROVEMENTS AND FUTURE AREAS OF RESEARCH

Careful theoretical planning of a scientific system may produce a final set of apparatus and analytical technique that is near-optimal from both data acquisition and operator convenience viewpoints. More often it is only when the planned apparatus and techniques are put into practise that improvements in the functioning of the system become obvious. Although the designed apparatus and analytical methods used in this work have demonstrated good agreement of the rank order laboratory permeation data from various topical formulations with that observed *in vivo*, scope for improving the methodology or increasing the sensitivity of the system has become apparent in several areas.

From a general overview of all the results and discussion it is proposed that any modifications made to the *in vitro* techniques so that they more closely simulate the blanching trial methodology will produce even closer correlation of the two sets of data. The blanching assay has been proven a valid qualitative technique for estimating percutaneous corticosteroid absorption. By improving on the *in vitro* diffusion cell design it may be possible to give some correlating, quantitative assessment of the absorption, without the need for drug-serum concentration analysis.

One aspect where the *in vitro* and *in vivo* methodologies differ markedly is the quantity of drug formulation applied to the donor surface of the membrane. A finite amount of product is applied to the skin in the blanching assay and remains in contact with the stratum corneum for a specific period. In contrast, an infinite amount of formulation is applied to the membrane *in vitro* and remains in contact for the duration of the experiment. It should be possible to apply the same relative quantity of formulation per unit area of *in vitro* membrane as is employed in the blanching protocol and this standard, finite dose of donor formulation should produce even closer correlation of the laboratory data with the *in vivo* data than has been experienced in this investigation.

Furthermore, as it has been demonstrated *(2)* that sequential treatment of the *in vitro* membrane may be undertaken, it may be possible to remove residual formulation from the membrane surface after a specific contact time, corresponding to the contact time used in the blanching trials. In this manner not only would the same quantity of formulation be applied to unit surface area of membrane as is used *in vivo*, but the product would be left

in contact with the membrane for the same period.

In addition, this improved finite dose methodology would be more capable of simulating the occluded and unoccluded blanching modes as there would be a relatively thin film of aqueous formulation on the surface of the *in vitro* membrane which would not hydrate the barrier to the same extent as experienced with infinite vehicle doses. These investigations have demonstrated that the relatively thick layer of formulation applied to the membrane provides copious moisture at the interface and may thereby hydrate the stratum corneum. If a relatively thin film of vehicle was spread over the membrane surface then moisture may evaporate from the exposed surface of the formulation into the ambient environment of the diffusion cell donor chamber, and thereby relatively less moisture would be available to hydrate the membrane. The presence or absence of an occlusive covering over the donor formulation would simulate the blanching assay conditions of occlusion or nonocclusion by preventing or allowing this moisture to escape to the atmosphere. However, complete simulation of the two application modes is impossible as there is endogenous water present *in vivo* which may be trapped in the skin by the vehicle, whereas *in vitro* the water for hydration is supplied solely by the applied formulation. Therefore, it is anticipated that nonaqueous formulations (ointments for example) may not simulate the occluded and unoccluded modes as closely as aqueous products.

Thus, by optimizing the donor vehicle contact with the membrane, the mass of formulation applied and the period of contact would be standardized for the two assay methods, and, furthermore, the simulation of the occluded or unoccluded application modes would be possible. Closer correlation of the two sets of data should be obtained under these conditions.

Allied to the improvements in the data acquisition described above, there would be a reduction in the length of each permeation experiment. Drug diffusion has been monitored for eight days through the animal media in this investigation, however this period would be undesirable for routine analysis. The blanching assays are usually conducted over 32 hours with the most important data acquisition occurring within the first 18 hours of observation. With improved diffusion cell methodology it seems highly feasible that *in vitro* experiments of this length should be able to provide sufficient, accurate data for meaningful comparative analysis.

One possibility in this regard is to increase the *in vivo* contact time of the formulations with the forearm skin. By allowing the *in vitro* contact period to be equally lengthy, higher concentrations of permeant would accumulate in the receptor chamber which would be easier to quantify by the

available techniques. However, the degree of blanching that can be induced at the application sites is finite and will be generated by a finite mass of corticosteroid reaching the dermal blanching receptors. Any excess in drug over this finite amount cannot manifest a greater degree of blanching and would not be visually detected *in vivo*. On the other hand, the *in vitro* permeation of these molecules would be quantitatively monitored by the analytical techniques employed. There is, therefore, an optimum *in vivo* contact time for the formulation and skin so that blanching saturation is not attained. This period appears to be between six and nine hours, depending on the potency of the corticosteroid tested. From a volunteer viewpoint the adhesive labels, guard and tape on the forearms becomes uncomfortable after a few hours and this would also discourage lengthening the contact time.

If the contact time of the finite dose of the donor formulation with the *in vitro* membrane cannot readily be lengthened, then the sensitivity of the analytical method used to monitor drug permeation must be increased so that the permeant mass entering the receptor chamber in the first 24 hours of experimentation may be fully characterized. This may be achieved by employing scintillation counting of radiolabelled corticosteroids that are formulated extemporaneously. This method has the added advantage of allowing immediate assay of the permeant mass on withdrawal of the sample from the receptor chamber. In contrast, samples are usually stored until sufficient have accumulated to warrant operation and calibration of the high-performance liquid chromatograph. On the other hand, even though the liquid chromatography method has a lower limit of permeant detection, it is the only feasible technique readily available for monitoring drug permeation from nonradiolabelled proprietary formulations.

Obviously, if the permeation surface area to receptor chamber volume ratio could be improved the sensitivity of either analytical method would be equally increased. In this investigation the diffusion half-cells had a volume of 10 ml, there seems to be no reason why their volume could not be reduced to 2 ml, for example, while retaining the same membrane surface area and efficient mixing hydrodynamics. The sensitivity of the analytical techniques would thus be improved approximately fivefold as the permeant concentrations in the receptor chamber would be that much greater per unit time after initiation of the experiment. This would allow complete characterization of this initial permeation period which would be extremely useful in the estimation of correlation with *in vivo* blanching data. The advantages of this early period characterization have been seen with the hydrocortisone formulations.

A number of other variables in the methodology may be investigated in an attempt to achieve better *in vivo–in vitro* data correlation. This investigation has employed the human blanching assay as the *in vivo* method of estimating drug absorption. It has been suggested that the blanching elicited after topical corticosteroid administration is generated by a small percentage of the drug diffusing rapidly via the shunt pathways, and does not necessarily reflect the absorption that occurs at steady-state. It is possible, therefore, that other corticosteroid bioassays *(276)*, or the monitoring of serum drug concentrations after topical administration, may produce data that correlates more closely with the observed *in vitro* permeation rates.

Similarly, other biological or polymer membranes, or different receptor chamber solvents, may be investigated. It is possible that a different membrane, when used in this cell design, may produce *in vitro* permeation results that agree more closely with *in vivo* bioassay data, and receptor solvents other than isopropyl myristate may be more appropriate for the monitoring of betamethasone 17-valerate permeation.

There are other small facets of the *in vitro* methodology that may be improved upon. The method of weanling pig stratum corneum preparation has been a noticeable shortcoming in the methodology of this investigation. It has been suggested that dermatome sectioning of this medium may produce more uniform strata that should exhibit less *in vitro* variance in their permeability. By using these uniform membranes it should be more facile to make significant distinctions between the drug release rates from similar formulations as the standard deviations of the mean permeation values should be proportionately smaller.

One factor of the blanching trial methodology that could be improved upon is the method of semisolid formulation application to the sites on the forearm. Extrusion from a syringe does not appear to be the ideal method. A possible improvement may be to spread the vehicles directly onto the sites using the tip of a glass rod, as is generally practiced in several laboratories. Preliminary investigations recording the before and after spreading mass of 7 x 7 mm paper squares using a cream and small tipped glass rod yield an average formulation mass applied of 2.634 mg (SD = 0.409 mg) as compared to the 3.03 mg average (SD = 0.710 mg) reported by Magnus *et al. (291)* for syringe extrusion. Glass rod spreading, therefore, appears to be one method of accomplishing more precise formulation application, obviously the tip of the glass rod may be enlarged if a greater mass is to be applied.

These are all factors that may be investigated in the future in an attempt to attain closer agreement of the *in vitro* and *in vivo* permeation

data. Generally the results of these investigations are promising in that they confirm the feasibility of using a well designed *in vitro* diffusion cell to estimate the rate and extent of betamethasone 17-valerate permeation through human and animal skin. This system has been shown especially useful for estimating comparative rank order betamethasone 17-valerate permeation from similar formulations, and should, therefore, be applicable in the future to the testing of new corticosteroid formulations for drug release potential.

The applicability of this cell design to the monitoring of hydrocortisone has also been demonstrated. It is therefore proposed that, with the improvements suggested above, this *in vitro* methodology may be applied to the monitoring of transdermal absorption of all classes of corticosteroid, and possibly other classes of drugs. Such a system would be extremely useful in any laboratory studying percutaneous absorption, especially in view of the current world-wide interest in transdermal drug delivery.

APPENDIX 1: Topical Preparations Used in this Study

The preparations employed were rated at a label concentration of 0.1% betamethasone (as the 17-valerate ester), or 0.5% hydrocortisone.

BETAMETHASONE 17-VALERATE FORMULATIONS

BETNOVATE FORMULATIONS

Manufacturer: Glaxo (Pty) Ltd., Manchester Rd., Wadeville, S A.

Assay Values:	Cream	99.02% (SD = 0.49%)
(% purity)	Lotion	93.53% (SD = 3.24%)
	OIntment	104.50% (SD = 1.08%)
	Scalp Application	121.61% (SD = 1.36%)

CELESTODERM-V FORMULATIONS

Manufacturer: Scherag (Pty) Ltd., Electron Ave., Isando, S A.

Assay Values:	Cream	99.06% (SD = 1.30%)
(% purity)	Lotion	101.79% (SD = 3.01%)
	Ointment	88.26% (SD = 4.70%)

PERSIVATE FORMULATIONS

Manufacturer: Lennon Limited, Fairclough Rd., Port Elizabeth, S A.

Assay Values:	Cream	109.27% (SD = 1.33%)
(% purity)	Ointment	100.52% (SD = 5.08%)

HYDROCORTISONE FORMULATIONS

CUTADERM CREAM

Manufacturer: Scherag (Pty) Ltd., Electron Ave., Isando, S A.

Assay value: 121.27% (SD = 3.82%)

LENNON CREAM

Manufacturer: Lennon Limited, Fairclough Rd., Port Elizabeth, S A.

Assay value: 128.86% (SD = 1.18%)

APPENDIX 2: Listing of "Superblanch" Programme

```
5  REM  ##################### SUPERBLANCH ####################
10  REM  LINES 60 TO 200 ESTABLISH THE PRIMARY MENU
20  REM  LINES 220 TO 2780 ENTER THE LOGISTICAL INFORMATION
30  REM  LINES 2800 TO 4880 ENTER THE ACTUAL BLANCHING SCORES
40  REM  LINES 4900 TO 9810 CARRY OUT THE STATS ANALYSIS
50  REM  LINES 9830 TO 9990 SHUT DOWN
60  HOME : PRINT "THIS PROGRAMME STORES AND STATISTICALLY"
70  VTAB 3: HTAB 3: PRINT "ANALYSES DATA FROM BLANCHING TRIALS"
80  VTAB 6: HTAB 3: PRINT "SELECT AN OPTION BY TYPING A NUMBER"
90  VTAB 8: HTAB 6: PRINT "FOLLOWED BY THE RETURN BUTTON"
100  VTAB 12: INVERSE : PRINT "1.  START UP OF NEW TRIAL-INITIALIZE NEW";
110  PRINT "    DATA DISK;INPUT APPLICATION INFO.    "
120  PRINT "2.  RECORD / EDIT BLANCHING SCORES ON    ";
130  PRINT "    EXISTING TRIAL DATA DISK             "
140  PRINT "3.  STATISTICAL ANALYSIS OF DATA ALREADY";
150  PRINT "    ENTERED                              "
160  PRINT "4. TERMINATE EDITING / SHUT DOWN         ": NORMAL
170  CLEAR : LET S =  - 16336: LET Z$ =  CHR$ (4): GOSUB 9980: REM  SOUND & CTRL-D
180  INPUT A: REM  MENU OPTION
190  ON A GOTO 220,2800,4900,9830
200  PRINT "INVALD INPUT:OPTIONS 1,2,3 OR 4": GOTO 170
210  REM ################################################## 1
220  HOME : VTAB 5: HTAB 2: PRINT "THIS SECTION DEALS WITH THE OPENING OF"
230  VTAB 7: HTAB 3: PRINT "NEW DATA FILES AND THE RECORDING OF"
240  VTAB 9: HTAB 2: PRINT "APPLICATION AND OBSERVATION INFORMATION"
250  VTAB 12: HTAB 5: PRINT "THIS SECTION IS NOT FOR RECORDING"
260  VTAB 14: HTAB 10: PRINT "ACTUAL BLANCHING SCORES": GOSUB 9980: GOSUB 9940
270  INPUT W$: IF W$ <  > "C" THEN  GOTO 10
280  HOME : VTAB 10: HTAB 3: PRINT "INSERT A DISK INTO DRIVE 2 FOR DATA"
290  VTAB 12: HTAB 3: PRINT "STORAGE  NB.PRESENT CONTENTS OF THIS"
300  VTAB 14: HTAB 11: PRINT "DISK WILL BE ERASED": GOSUB 9980: GOSUB 9940
310  INPUT W$: IF W$ <  > "C" THEN  GOTO 10
320  HOME : VTAB 4: HTAB 3: PRINT "TYPE IN A HEADING FOR YOUR DATA DISK"
330  VTAB 6: HTAB 4: PRINT "IDENTIFYING IT FOR LATER REFERENCE"
340  VTAB 9: HTAB 4: PRINT "EG. BETNOVATE CREAM TRIAL- 15/8/85"
350  VTAB 11: HTAB 5: PRINT "HEADING CAN BE 30 CHARACTERS LONG"
360  VTAB 13: HTAB 9: PRINT "PRESS RETURN ONLY AT END"
370  VTAB 15: HTAB 11: INVERSE : FLASH : PRINT "NO COLONS OR COMMAS": NORMAL : GOSUB 9980
380  VTAB 17: HTAB 9: INVERSE : PRINT "'R'-RETURN TO MAIN MENU"
390  VTAB 21: HTAB 2: PRINT "--------30 CHARACTERS---------": NORMAL
400  INPUT H1$: IF H1$ = "R" THEN  GOTO 10: REM  TRIAL HEADING
410  HOME : VTAB 5: HTAB 11: INVERSE : PRINT "CHECK YOUR ENTRY": NORMAL
420  VTAB 10: HTAB 5: PRINT H1$: GOSUB 9980: GOSUB 9960
430  INPUT W$: IF W$ <  > "C" THEN  GOTO 320
440  HOME : VTAB 5: HTAB 5: PRINT H1$: GOSUB 9980: PRINT Z$;"MON,C,I,0"
450  VTAB 15: HTAB 5: PRINT "INITIALIZING DATA DISK-PLEASE WAIT"
460  PRINT Z$;"OPEN TRIALNAME,D1": REM  SAVING TRIALNAME ON D1
470  PRINT Z$;"WRITE TRIALNAME"
480  PRINT H1$
490  PRINT Z$;"CLOSE"
500  PRINT Z$;"BLOAD CHAIN,A520"
510  CALL 520"GREETING": REM   RUNNING GREETING PROGRAMME
520  LET Z$ =  CHR$ (4):S =  - 16336: REM  RETURNING FROM GREETING
530  PRINT Z$;"OPEN TRIALNAME,D1": REM  PRINTING TRIALNAME ON D2
540  PRINT Z$;"READ TRIALNAME"
550  INPUT H1$: PRINT Z$;"CLOSE"
560  PRINT Z$;"OPEN";H1$;",D2"
570  PRINT Z$;"CLOSE": PRINT Z$;"NOMON,C,I,0"
```

```
580  HOME : VTAB 3: HTAB 5: INVERSE : PRINT "TYPE IN NUMBER OF OBSERVERS": NORMAL : PRINT : GOSUB 9980
590  INPUT O1:: REM  #/ OBSERVERS
600  VTAB 7: PRINT "TYPE IN ONE IDENTIFYING INITIAL FOR EACH"
610  VTAB 9: HTAB 8: PRINT "OBSERVER: EG. JOHN='J'": VTAB 12
620  FOR A = 1 TO O1
630  FOR BEEP = 1 TO 40:SOUND =  PEEK (S): NEXT BEEP
640  INVERSE : PRINT "OBSERVER: ";A: NORMAL
650  INPUT O$(A): REM  OBSERVER'S INITIAL
660  IF  LEN (O$(A)) <  > 1 GOTO 680
670  PRINT : PRINT : NEXT A: GOTO 710
680  HOME : VTAB 15: PRINT "TOO MANY INITIALS/DUPLICATION-TRY AGAIN"
690  GOSUB 10000
700  GOTO 580
710  FOR A = 1 TO (O1 - 1)
720  FOR B = (A + 1) TO O1
730  IF O$(A) = O$(B) THEN  GOTO 680
740  NEXT B
750  NEXT A
760  HOME : FLASH : VTAB 3: HTAB 12: PRINT "CHECK YOUR ENTRY": NORMAL
770  VTAB 7: FOR A = 1 TO O1
780  HTAB 9: PRINT "OBSERVER ";A;" INITIAL: ";O$(A): PRINT
790  NEXT A: GOSUB 9980: GOSUB 9960
800  INPUT W$: IF W$ <  > "C" THEN  GOTO 580
810  GOSUB 9990
820  PRINT Z$;"OPEN OBSERVERS,D2"
830  PRINT Z$;"WRITE OBSERVERS"
840  PRINT O1
850  FOR A = 1 TO O1
860  PRINT O$(A)
870  NEXT
880  PRINT Z$;"CLOSE"
890  HOME : INVERSE : VTAB 5: HTAB 1: PRINT "TYPE IN NUMBER OF APPLICATION PATTERNS"
900  VTAB 7: HTAB 17: PRINT "USED": GOSUB 9980
910  VTAB 10: INPUT P1:: REM   #/ PATTERNS
920  VTAB 16:: HTAB 1: PRINT "TYPE IN NUMBER OF SITES PER APPLICATION"
930  VTAB 18: HTAB 16: PRINT "PATTERN": GOSUB 9980
940  VTAB 20: INPUT P2: NORMAL : REM  #/SITES PER PATTERN
950  HOME : VTAB 5: HTAB 12: INVERSE : PRINT "CHECK YOUR ENTRY": NORMAL
960  VTAB 8: HTAB 3: PRINT "NUMBER OF APPLICATION PATTERNS: ";P1
970  VTAB 11: HTAB 4: PRINT "NUMBER OF SITES PER PATTERN: ";P2: GOSUB 9980: GOSUB 9960
980  INPUT W$: IF W$ <  > "C" THEN  GOTO 890
990  DIM PSITE$(P1)
1000  HOME : VTAB 4: HTAB 3: PRINT "TYPE IN THE PREPARATION APPLICATION"
1010  VTAB 6: HTAB 7: PRINT "LAYOUT FOR EACH PATTERN.EG."
1020  VTAB 9: PRINT "SITE NUMBER: "; SPC( 7);"123456789012 ETC."
1030  PRINT "PREPARATION APPLIED:ACDBCABD ETC."
1040  VTAB 13: PRINT "REMEMBER:PREPARATIONS ARE CODED A,B,C,D"
1050  VTAB 15: HTAB 10: PRINT "SITES ARE NUMBERED 1,2,3,4 ETC."
1060  VTAB 17: HTAB 10: PRINT "CHARTS ARE CODED 1,2,3,4 ETC."
1070  VTAB 20: HTAB 10: INVERSE : PRINT "PRESS 'C' TO CONTINUE": NORMAL : GOSUB 9980
1080  GET W$: IF W$ <  > "C" THEN  GOTO 10
1090  FOR A = 1 TO P1
1100  HOME : VTAB 5: HTAB 10: PRINT "APPLICATION CHART ";A: GOSUB 9980
1110  VTAB 9: HTAB 2: PRINT "SITE NUMBERS:"
1120  VTAB 11: HTAB 2: PRINT "12345678901234 ETC."
1130  VTAB 13: HTAB 2: INVERSE : PRINT "--12 SITES--",: FLASH : PRINT "TYPE IN PREP.CODES ACB..": NORMAL
1140  VTAB 14: INPUT PSITE$(A)
1150  IF  LEN (PSITE$(A)) <  > P2 THEN  GOTO 1190
1160  FOR B = 1 TO P2: IFO
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```
1170  NEXT B
1180  NEXT A: GOTO 1220
1190  HOME : VTAB 10: HTAB 8: PRINT P2;" CHARACTERS NOT ENTERED"
1200  VTAB 12: HTAB 16: PRINT "TRY AGAIN"
1210  GOSUB 10000: GOTO 1100
1220  HOME : VTAB 2: HTAB 11: INVERSE : PRINT "CHECK YOUR ENTRY": NORMAL
1230  VTAB 5: HTAB 2: PRINT "SITE NUMBER:    12345678901234 ETC.": VTAB 7
1240  FOR A = 1 TO P1: REM  #/ CHARTS
1250  PRINT "CHART NUMBER: ";A;" -";PSITE$(A): PRINT : PRINT
1260  NEXT A: GOSUB 9980: GOSUB 9960
1270  INPUT W$: IF W$ <  > "C" THEN  GOTO 1090
1280  GOSUB 9990
1290  PRINT Z$;"OPEN PATTERNS,D2"
1300  PRINT Z$;"WRITE PATTERNS"
1310  PRINT P1: PRINT P2
1320  FOR A = 1 TO P1
1330  PRINT PSITE$(A)
1340  NEXT A
1350  PRINT Z$;"CLOSE"
1360  HOME : VTAB 2: INVERSE : PRINT "TYPE IN NUMBER OF COMPARISONS PER CHART": NORMAL
1370  GOSUB 9980: VTAB 4: INPUT C1: REM  #/COMPARISONS PER CHART
1380  IF C1 > 0 THEN  GOTO 1490
1390  VTAB 10: HTAB 12: INVERSE : PRINT "CHECK YOUR ENTRY": NORMAL : PRINT
1400  HTAB 10: FLASH : PRINT "))";: NORMAL : PRINT " NO COMPARISONS ";: FLASH : PRINT "((": NORMAL
1410  GOSUB 9980: GOSUB 9960
1420  INPUT W$: IF W$ <  > "C" THEN  GOTO 1360
1430  GOSUB 9990
1440  FOR A = 1 TO P1
1450  PRINT Z$;"OPEN COMPS";A;",L10,D2"
1460  PRINT Z$;"WRITE COMPS";A;",R1"
1470  PRINT "1/2": PRINT Z$;"CLOSE"
1480  NEXT A: GOTO 1790
1490  VTAB 7: HTAB 4: PRINT "FOR EACH CHART, TYPE IN THE NUMBERS"
1500  VTAB 9: HTAB 3: PRINT "REPRESENTING THE SITES TO BE COMPARED"
1510  VTAB 12: HTAB 4: PRINT "NUMBERS TO BE SEPARATED BY A SLASH"
1520  VTAB 14: PRINT "EG.  CHART NUMBER: 1"
1530  VTAB 16: HTAB 6: PRINT "1/2 (RETURN)"
1540  HTAB 6: PRINT "3/2 (RETURN)"
1550  HTAB 5: PRINT "11/12(RETURN)  ETC."
1560  VTAB 20: HTAB 12: INVERSE : PRINT "'C' TO CONTINUE"
1570  VTAB 22: HTAB 2: PRINT "'R' TO RE-TYPE NUMBER OF COMPARISONS": NORMAL
1580  GOSUB 9980: INPUT W$: IF W$ <  > "C" THEN  GOTO 1360
1590  DIM C$(C1): FOR A = 1 TO P1
1600  HOME : INVERSE : VTAB 3: HTAB 11: FLASH : PRINT "TYPE IN COMPARISONS": NORMAL : GOSUB 9980
1610  VTAB 6: INVERSE : PRINT "CHART NUMBER: ";A: NORMAL : VTAB 8
1620  FOR B = 1 TO C1
1630  INPUT C$(B): REM  SITES TO BE COMPARED
1640  NEXT B
1650  HOME : VTAB 2: HTAB 12: INVERSE : PRINT "CHECK YOUR ENTRY": NORMAL : GOSUB 9980
1660  VTAB 4: PRINT "CHART NUMBER: ";A: VTAB 6
1670  FOR B = 1 TO C1
1680  HTAB 15: PRINT C$(B)
1690  NEXT B: GOSUB 9960
1700  INPUT W$: IF W$ <  > "C" THEN  GOTO 1600
1710  GOSUB 9990
1720  PRINT Z$;"OPEN COMPS";A;",L10,D2"
1730  FOR B = 1 TO C1
1740  PRINT Z$;"WRITE COMPS";A;",R";B
```

```
1750  PRINT C$(B)
1760  NEXT B
1770  PRINT Z$;"CLOSE"
1780  NEXT A
1790  HOME : VTAB 5: HTAB 2: INVERSE : PRINT "TYPE IN NUMBER OF PREPARATIONS USED"
1800  VTAB 7: HTAB 9: PRINT "IN THE TRIAL";: FLASH : PRINT "(MAX. 12)": NORMAL
1810  GOSUB 9980: INPUT R1: IF R1 > 12 THEN  GOTO 1790: REM  #/ PREPS USED
1820  IF R1 < 1 THEN  GOTO 1790
1830  VTAB 12: HTAB 12: INVERSE : PRINT "CHECK YOUR ENTRY": NORMAL : GOSUB 9980
1840  VTAB 15: HTAB 10: PRINT R1;" PREPARATIONS USED"
1850  GOSUB 9960
1860  INPUT W$: IF W$ <  > "C" THEN  GOTO 1790
1870  DIM PREP$(R1)
1880  HOME : VTAB 3: HTAB 3: PRINT "TYPE IN THE NAME OF EACH PREPARATION"
1890  VTAB 5: HTAB 5: PRINT "AGAINST ITS LETTER CODE AS USED"
1900  VTAB 7: HTAB 7: PRINT "IN THE APPLICATION PATTERNS"
1910  VTAB 10: HTAB 7: PRINT "EG. PREP.A = BETNOVATE CREAM"
1920  LET R1$ = "ABCDEFGHIJKL": VTAB 13
1930  FOR A = 1 TO R1
1940  LET R2$ =  MID$ (R1$,A,1)
1950  INVERSE : PRINT "PREPARATION ";R2$: NORMAL : PRINT : GOSUB 9980
1960  INPUT PREP$(A): PRINT : REM  PREP NAME
1970  NEXT A
1980  HOME : VTAB 2: HTAB 12: INVERSE : PRINT "CHECK YOUR ENTRY": NORMAL : VTAB 5
1990  FOR A = 1 TO R1
2000  LET R2$ =  MID$ (R1$,A,1)
2010  PRINT "PREP. ";R2$;" = ";PREP$(A): PRINT
2020  NEXT A: PRINT : GOSUB 9980: GOSUB 9960
2030  INPUT W$: IF W$ <  > "C" THEN  GOTO 1880
2040  GOSUB 9990
2050  PRINT Z$;"OPEN PREPARATIONS,D2"
2060  PRINT Z$;"WRITE PREPARATIONS"
2070  FOR A = 1 TO R1
2080  PRINT PREP$(A)
2090  NEXT
2100  PRINT Z$;"CLOSE"
2110  HOME : VTAB 5: HTAB 4: INVERSE : PRINT "TYPE IN NUMBER OF VOLUNTEERS USED"
2120  VTAB 7: HTAB 14: PRINT "IN THE TRIAL": NORMAL : GOSUB 9980
2130  VTAB 9: INPUT V1: IF V1 < 1 THEN  GOTO 2110: REM  #/ VOLUNTEERS
2140  VTAB 12: HTAB 2: INVERSE : PRINT "TYPE IN NUMBER OF OCCASIONS ON WHICH"
2150  VTAB 14: HTAB 7: PRINT "BLANCHING DATA WAS RECORDED": NORMAL : GOSUB 9980
2160  VTAB 16: INPUT T1: IF T1 < 1 THEN  HOME : GOTO 2140: REM  #/ TIME INTERVALS
2170  HOME : VTAB 5: HTAB 12: INVERSE : PRINT "CHECK YOUR ENTRY": NORMAL : GOSUB 9980
2180  VTAB 8: HTAB 4: PRINT "NUMBER OF VOLUNTEERS: ";V1
2190  VTAB 11: HTAB 4: PRINT "NUMBER OF DATA COLLECTION TIMES: ";T1
2200  GOSUB 9960
2210  INPUT W$: IF W$ <  > "C" THEN  GOTO 2110
2220  GOSUB 9990
2230  PRINT Z$;"OPEN MISC,D2"
2240  PRINT Z$;"WRITE MISC"
2250  PRINT V1: PRINT T1: PRINT H1$: PRINT P1: PRINT P2: PRINT C1: PRINT R1: PRINT O1
2260  PRINT Z$;"CLOSE": DIM T$(T1)
2270  HOME : VTAB 2: HTAB 3: PRINT "THERE ARE ";T1;" DATA RECORDING PERIODS"
2280  VTAB 4: HTAB 12: PRINT "DURING THE TRIAL"
2290  VTAB 7: HTAB 2: PRINT "FOR EACH PERIOD TYPE IN THE NUMBER OF"
2300  VTAB 9: PRINT "HOURS AFTER PREPARATION APPLICATION THAT"
2310  VTAB 11: HTAB 8: PRINT "THE BLANCHING WAS READ"
2320  VTAB 14: PRINT "EG. PERIOD 1: ";: INVERSE : FLASH : PRINT "7";: NORMAL : PRINT " HOURS AFTER APPLICATION"
2330  VTAB 16: HTAB 5: PRINT "PERIOD 2: ";: FLASH : PRINT "8";: NORMAL : PRINT " HOURS.....ETC."
2340  VTAB 19: HTAB 10: FLASH : PRINT "TYPE IN NUMBER OF HOURS": NORMAL
```

```
2350  FOR A = 1 TO T1
2360  INVERSE : PRINT "PERIOD ";A: NORMAL : PRINT
2370  FOR BEEP = 1 TO 40:SOUND =  PEEK (S): NEXT BEEP
2380  INPUT T$(A): PRINT
2390  NEXT A
2400  HOME : VTAB 2: HTAB 11: INVERSE : PRINT "CHECK YOUR ENTRY": NORMAL : PRINT
2410  FOR A = 1 TO T1
2420  PRINT "PERIOD ";A;": ";T$(A);" HOURS"
2430  NEXT A: GOSUB 9980: GOSUB 9960
2440  INPUT W$: IF W$ (  ) "C" THEN  GOTO 2270
2450  GOSUB 9990
2460  PRINT Z$;"OPEN TIMES,L5,D2"
2470  FOR A = 1 TO T1
2480  PRINT Z$;"WRITE TIMES,R";A
2490  PRINT T$(A)
2500  NEXT
2510  PRINT Z$;"CLOSE"
2520  HOME : VTAB 12: HTAB 10: PRINT "CREATING DATA FILES"
2530  VTAB 15: HTAB 3: PRINT "THIS PROCESS TAKES SEVERAL MINUTES"
2540  VTAB 18: HTAB 14: PRINT "PLEASE WAIT": GOSUB 9980
2550  PRINT Z$;"MON,C,I,0": LET X$ = "X"
2560  IF  LEN (X$) (  ) T1 THEN  LET X$ = X$ + "X": GOTO 2560
2570  FOR A = 1 TO 01
2580  FOR C = 1 TO V1
2590  PRINT Z$;"OPEN";O$(A);C;",L";(P2 + C1 + 7);",D2"
2600  PRINT Z$;"WRITE";O$(A);C;",R";(T1 + 1)
2610  PRINT X$
2620  PRINT Z$;"WRITE";O$(A);C;",R";((T1 + 1) * 2)
2630  PRINT X$
2640  PRINT Z$;"CLOSE"
2650  NEXT C
2660  NEXT A
2670  PRINT Z$;"LOCK OBSERVERS,D2": PRINT Z$;"LOCK GREETING,D2"
2680  PRINT Z$;"LOCK PATTERNS,D2": PRINT Z$;"LOCK ";H1$;",D2"
2690  FOR A = 1 TO P1: PRINT Z$;"LOCK COMPS";A;",D2": NEXT
2700  PRINT Z$;"LOCK PREPARATIONS,D2"
2710  PRINT Z$;"LOCK MISC,D2"
2720  PRINT Z$;"LOCK TIMES,D2"
2730  PRINT Z$;"NOMON,C,I,0"
2740  HOME : VTAB 10: HTAB 9: FLASH : PRINT "ALL INFORMATION ENTERED"
2750  VTAB 16: HTAB 4: PRINT "PRESS 'RETURN' KEY FOR MAIN MENU": NORMAL : VTAB 23
2760  FOR A = 1 TO 5
2770  FOR BEEP = 1 TO 50:SOUND =  PEEK (S): NEXT BEEP: FOR BEEP = 1 TO 50:SOUND =  PEEK (S) -  PEEK (S): NEXT
2780  NEXT A: INPUT W$: GOTO 10
2790  REM ################################################## 2
2800  HOME : VTAB 2: HTAB 2: PRINT "THIS SECTION DEALS WITH THE RECORDING"
2810  VTAB 4: HTAB 2: PRINT "AND STORAGE OF ACTUAL BLANCHING SCORES"
2820  VTAB 6: HTAB 4: PRINT "ONTO A PRE-INITIALIZED DATA DISK"
2830  VTAB 9: HTAB 2: PRINT "IF A DATA DISK HAS NOT BEEN INITIALIZED"
2840  VTAB 11: HTAB 4: PRINT "FOR THIS TRIAL RETURN AND COMPLETE"
2850  VTAB 13: HTAB 4: PRINT "SECTION 1 OF THIS PROGRAMME FIRST"
2860  GOSUB 9980: GOSUB 9940
2870  INPUT W$: IF W$ (  ) "C" THEN  GOTO 10
2880  HOME : VTAB 4: HTAB 9: PRINT "DO YOU WISH TO USE THE": PRINT : PRINT : HTAB 7: FLASH
2890  HTAB 12: PRINT "*)  YES / NO  (*": GOSUB 9980 : PRINT "**) VOICE INPUT MODULE (**"
2900  INPUT W$: IF  MID$ (W$,1,1) (  ) "Y" THEN  GOTO 2960
2910  VTAB 16: HTAB 1: PRINT "ARE YOU SURE YOU WANT TO USE THE MODULE": GOSUB 9980: PRINT "*)  YES / NO  (*"
2920  INPUT W$: IF  MID$ (W$,1,1) (  ) "Y" THEN  GOTO 2880
2930  HOME : VTAB 10: HTAB 5: PRINT "LOADING VOICE INPUT PROGRAMMES": GOSUB 9980:PRINT "PLEASE WAIT"
2940  PRINT Z$;"BLOAD CHAIN,A520,D1"
2950  CALL 520"AVIN"
2960  HOME : VTAB 10: HTCC$ = "U" THEN  PRINT Z$;"WRITE";OB$;V2;",R";((T1 + 1) * 2)
```

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2970 VTAB 12: HTAB 12: PRINT "INTO DISK DRIVE 2": GOSUB 9980: GOSUB 9940
2980 INPUT W$: IF W$ ( ) "C" THEN  GOTO 10
2990 HOME : VTAB 12: HTAB 17: PRINT "WAIT"
3000 PRINT Z$;"OPEN MISC,D2"
3010 PRINT Z$;"READ MISC"
3020 INPUT V1,T1,H1$,P1,P2,C1,R1,O1
3030 PRINT Z$;"CLOSE"
3040 PRINT Z$;"OPEN OBSERVERS,D2"
3050 PRINT Z$;"READ OBSERVERS"
3060 INPUT O1
3070 FOR A = 1 TO O1
3080 INPUT O$(A): NEXT
3090 PRINT Z$;"CLOSE"
3100 HOME : VTAB 8: HTAB 7: PRINT "THIS DATA DISK IS LABELLED:"
3110 VTAB 12: HTAB 7: PRINT H1$: GOSUB 9980
3120 VTAB 18: HTAB 12: INVERSE : PRINT "'C' TO CONTINUE"
3130 VTAB 20: HTAB 8: PRINT "'R' TO REPLACE DATA DISK": NORMAL
3140 INPUT W$: IF W$ ( ) "C" THEN  GOTO 2960
3150 HOME : HTAB 12: FLASH : PRINT "ENTER YOUR DATA": NORMAL
3160 VTAB 5: HTAB 5: PRINT "TYPE ";: FLASH : PRINT "EXIT";: NORMAL : PRINT " IF YOU DO NOT WISH TO"
3170 VTAB 7: HTAB 10: PRINT "ENTER ANY MORE DATA"
3180 VTAB 10: HTAB 2: PRINT "TYPE ";: FLASH : PRINT "CHECK";: NORMAL : PRINT " TO SEE HOW MANY INTERVALS"
3190 VTAB 12: HTAB 3: PRINT "ARE FILLED IN EACH VOLUNTEER'S FILE"
3200 VTAB 15: HTAB 8: PRINT "TYPE ";: FLASH : PRINT "HELP";: NORMAL : PRINT " IF YOU NEED IT!"
3210 VTAB 18: INVERSE : PRINT "OBSERVER'S INITIAL";: NORMAL : PRINT " ( ";
3220 FOR A = 1 TO O1: PRINT O$(A);: PRINT " ";: NEXT A
3230 PRINT ")   :";: GOSUB 9980
3240 INPUT OB$
3250 IF OB$ = "EXIT" THEN  GOTO 10
3260 IF OB$ = "CHECK" THEN  GOTO 4200
3270 IF OB$ = "HELP" THEN  GOTO 4490
3280 FOR A = 1 TO O1: IF OB$ = O$(A) THEN  GOTO 3310
3290 NEXT A
3300 HOME : VTAB 7: HTAB 12: FLASH : PRINT "INVALID INPUT": NORMAL : GOSUB 10000: GOTO 3210
3310 HOME : HTAB 12: INVERSE : FLASH : PRINT "ENTER YOUR DATA": NORMAL : PRINT
3320 INVERSE : PRINT "OBSERVER'S INITIAL";: NORMAL : PRINT " ( ";
3330 FOR A = 1 TO O1: PRINT O$(A);: PRINT " ";: NEXT A
3340 PRINT ")   : ";OB$: PRINT
3350 FOR BEEP = 1 TO 30:SOUND =  PEEK (S): NEXT BEEP
3360 INVERSE : PRINT "OCCLUDED OR UNOCCLUDED";: NORMAL : PRINT " (O OR U):";: INPUT OCC$
3370 IF OCC$ = "O" THEN  GOTO 3400
3380 IF OCC$ = "U" THEN  GOTO 3400
3390 PRINT : PRINT : GOSUB 10000: GOTO 3360
3400 FOR BEEP = 1 TO 30:SOUND =  PEEK (S): NEXT BEEP
3410 PRINT : INVERSE : PRINT "VOLUNTEER'S NUMBER";: NORMAL : PRINT " ( 1 TO ";V1;" ) :";: INPUT V2
3420 IF V2 ) V1 THEN  PRINT : GOSUB 10000: GOTO 3410
3430 IF V2 ( 1 THEN  PRINT : GOSUB 10000: GOTO 3410
3440 FOR BEEP = 1 TO 30:SOUND =  PEEK (S): NEXT BEEP
3450 PRINT : INVERSE : PRINT "DATA RECORDING PERIOD";: NORMAL : PRINT " (1 TO ";T1;: PRINT "):";: INPUT T2
3460 IF T2 ) T1 THEN  PRINT : GOSUB 10000: GOTO 3450
3470 IF T2 ( 1 THEN  PRINT : GOSUB 10000: GOTO 3450
3480 FOR BEEP = 1 TO 30:SOUND =  PEEK (S): NEXT BEEP
3490 PRINT : INVERSE : PRINT "APPLIC. PATTERN USED";: NORMAL : PRINT " (1 TO ";P1;")  :";: INPUT P3
3500 IF P3 ) P1 THEN  PRINT : GOSUB 10000: GOTO 3490
3510 IF P3 ( 1 THEN  PRINT : GOSUB 10000: GOTO 3490
3520 FOR BEEP = 1 TO 30:SOUND =  PEEK (S): NEXT BEEP
3530 PRINT : INVERSE : PRINT "ACTUAL BLANCHING SCORES";: NORMAL : PRINT " (0 TO 4)"
3540 PRINT : PRINT " ";: INVERSE : PRINT "--12 SITES--": NORMAL
3550 INPUT BLANCH$
3560 IF  LEN (BLANCH$) ( ) P2 THEN  GOSUB 10000: PRINT : GOTO 3530
3570 FOR A = 1 TO P2: LET X$ =  MID$ (BLANCH$,A,1)
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3580  IF X$ = "0" GOTO 3640
3590  IF X$ = "1" GOTO 3640
3600  IF X$ = "2" GOTO 3640
3610  IF X$ = "3" GOTO 3640
3620  IF X$ = "4" GOTO 3640
3630  PRINT : GOSUB 10000: GOTO 3530
3640  NEXT A
3650  IF C1 = 0 THEN  GOTO 3780
3660  FOR BEEP = 1 TO 30:SOUND =  PEEK (S): NEXT BEEP
3670  PRINT : INVERSE : PRINT "COMPARISONS";: NORMAL : PRINT " ( < > 0 OR = )": PRINT
3680  PRINT " ";: INVERSE : PRINT "----10----": NORMAL
3690  INPUT COMPAR$
3700  IF  LEN (COMPAR$) (  ) C1 THEN  GOSUB 10000: PRINT : GOTO 3670
3710  FOR A = 1 TO C1: LET X$ =  MID$ (COMPAR$,A,1)
3720  IF X$ = "(" THEN  GOTO 3770
3730  IF X$ = ")" THEN  GOTO 3770
3740  IF X$ = "0" THEN  GOTO 3770
3750  IF X$ = "=" THEN  GOTO 3770
3760  PRINT : GOSUB 10000: GOTO 3670
3770  NEXT A
3780  HOME : HTAB 12: FLASH : PRINT "CHECK YOUR DATA": GOSUB 9980
3790  VTAB 3: INVERSE : PRINT "OBSERVER'S INITIAL";: NORMAL : PRINT "      :";OB$: PRINT
3800  INVERSE : PRINT "OCCLUDED OR UNOCCLUDED";: NORMAL : PRINT "  :";OCC$
3810  PRINT : INVERSE : PRINT "VOLUNTEER'S NUMBER";: NORMAL : PRINT "      :";V2: PRINT
3820  INVERSE : PRINT "DATA RECORDING PERIOD";: NORMAL : PRINT "   :";T2: PRINT
3830  INVERSE : PRINT "APPLIC. PATTERN USED";: NORMAL : PRINT "    :";P3
3840  PRINT : INVERSE : PRINT "ACTUAL BLANCHING SCORES": NORMAL
3850  PRINT : PRINT " ";: INVERSE : PRINT "--12 SITES--": NORMAL
3860  PRINT " ";BLANCH$
3870  IF C1 = 0 THEN  PRINT : PRINT : GOTO 3910
3880  PRINT : INVERSE : PRINT "COMPARISONS": NORMAL : PRINT
3890  PRINT " ";: INVERSE : PRINT "----10----": NORMAL
3900  PRINT " ";COMPAR$: PRINT
3910  INVERSE : HTAB 4: PRINT "'C' TO CONTINUE / 'R' TO RE-ENTER": NORMAL
3920  INPUT W$: IF W$ <  > "C" THEN  GOTO 3150
3930  GOSUB 9990
3940  PRINT Z$;"OPEN";OB$;V2;",L";(P2 + C1 + 7);",B2"
3950  IF OCC$ = "O" THEN  PRINT Z$;"READ";OB$;V2;",R";(T1 + 1)
3960  IF OCC$ = "U" THEN  PRINT Z$;"READ";OB$;V2;",R";((T1 + 1) * 2)
3970  INPUT NUMBERS$: REM  CHECK X'S
3980  PRINT Z$;"CLOSE"
3990  LET N$ =  STR$ (T2)
4000  IF  LEN (N$) > 1 THEN  LET N$ =  MID$ (N$,2,1)
4010  IF T2 = 1 THEN  LET NUMBERS$ = N$ +  MID$ (NUMBERS$,2,( LEN (NUMBERS$) - 1)): GOTO 4040
4020  IF T2 = T1 THEN  LET NUMBERS$ =  LEFT$ (NUMBERS$,(T2 - 1)) + N$: GOTO 4040
4030  LET NUMBERS$ =  LEFT$ (NUMBERS$,(T2 - 1)) + N$ +  MID$ (NUMBERS$,(T2 + 1),( LEN (NUMBERS$) - T2))
4040  VTAB 12: HTAB 5: PRINT "OBSERVER: ";OB$: PRINT
4050  HTAB 5: PRINT "VOLUNTEER: ";V2: PRINT
4060  HTAB 5: PRINT "OCC. / UNOCC.: ";OCC$: PRINT : PRINT
4070  HTAB 10: PRINT "DATA INTERVALS ENTERED": PRINT
4080  HTAB 15: PRINT NUMBERS$
4090  PRINT Z$;"OPEN";OB$;V2;",L";(P2 + C1 + 7);",B2"
4100  IF OCC$ = "O" THEN  PRINT Z$;"WRITE";OB$;V2;",R";(T1 + 1)
4110  IF OCC$ = "U" THEN  PRINT Z$;"WRITE";OB$;V2;",R";((T1 + 1) * 2)
4120  PRINT NUMBERS$
4130  IF OCC$ = "O" THEN  PRINT Z$;"WRITE";OB$;V2;",R";T2
4140  IF OCC$ = "U" THEN  PRINT Z$;"WRITE";OB$;V2;",R";(T1 + 1 + T2)
4150  PRINT P3: REM  PATTERN USED
4160  PRINT BLANCH$: REM  SCORES
4170  IF C1 = 0 THEN  PRINT "0": GOTO 4190
4180  PRINT COMPAR$: REM  COMPARISONS
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4190  PRINT Z$;"CLOSE": GOTO 3150
4200  HOME : VTAB 2: HTAB 2: PRINT "THIS SECTION CHECKS WHICH OF THE DATA": PRINT
4210  HTAB 2: PRINT "RECORDING INTERVALS HAVE BEEN FILLED": PRINT
4220  PRINT "WITH INFORMATION IN EACH VOLUNTEERS FILE": PRINT : PRINT : GOSUB 9980
4230  INVERSE : FLASH : PRINT "TYPE IN";:PRINT "OBSERVER'S INITIAL";:INPUT OB$: PRINT
4240  FOR A = 1 TO O1
4250  IF OB$ = O$(A) THEN  GOTO 4280
4260  NEXT A
4270  HTAB 12: FLASH : PRINT "INVALID INITIAL": NORMAL : PRINT : GOSUB 10000: GOTO 4230
4280  FOR BEEP = 1 TO 40:SOUND =  PEEK (S): NEXT BEEP
4290  HTAB 11: INVERSE : PRINT "OCC. / UNOCC.";: NORMAL : INPUT OCC$: PRINT
4300  IF  MID$ (OCC$,1,1) = "O" THEN  GOTO 4330
4310  IF  MID$ (OCC$,1,1) = "U" THEN  GOTO 4330
4320  HTAB 12: FLASH : PRINT "INVALID INPUT": NORMAL : PRINT : GOSUB 10000: GOTO 4290
4330  FOR BEEP = 1 TO 40:SOUND =  PEEK (S): NEXT BEEP
4340  HTAB 11: INVERSE : PRINT "VOLUNTEER'S NUMBER";: NORMAL : INPUT V2: PRINT
4350  IF V2 ) V1 THEN  GOTO 4380
4360  IF V2 ( 1 THEN  GOTO 4380
4370  GOTO 4390
4380  HTAB 12: FLASH : PRINT "INVALID INPUT": NORMAL : PRINT : GOSUB 10000: GOTO 4340
4390  PRINT Z$;"OPEN";OB$;V2;",L";(P2 + C1 + 7);",D2"
4400  IF  MID$ (OCC$,1,1) = "O" THEN  PRINT Z$;"READ";OB$;V2;",R";(T1 + 1)
4410  IF  MID$ (OCC$,1,1) = "U" THEN  PRINT Z$;"READ";OB$;V2;",R";((T1 + 1) * 2)
4420  INPUT NUMBERS$
4430  PRINT Z$;"CLOSE"
4440  HTAB 15: PRINT NUMBERS$: PRINT : GOSUB 9980
4450  HTAB 2: PRINT "EACH 'X' REPRESENTS AN UNFILLED TIME": PRINT
4460  HTAB 10: PRINT "INTERVAL FROM 1 TO ";T1: PRINT
4470  INVERSE : HTAB 12: PRINT "'C' TO CONTINUE": NORMAL
4480  INPUT W$: GOTO 3150
4490  HOME : HTAB 8: PRINT "DATA RECORDED ON DISK": HTAB 8: PRINT "--------------------"
4500  PRINT : PRINT O1" OBSERVERS: INITIALS- ";: FOR A = 1 TO O1: PRINT O$(A);" ";: NEXT A: PRINT
4510  PRINT : PRINT V1;" VOLUNTEERS (NUMBERED 1 TO ";V1;")": PRINT
4520  PRINT "THERE ARE ";T1;" BLANCHING SCORE/COMPARISON": PRINT
4530  HTAB 10: PRINT "RECORDING INTERVALS": PRINT
4540  PRINT P1;" APPLICATION PATTERNS HAVE BEEN USED": PRINT
4550  PRINT "EACH PATTERN HAS ";P2;" APPLICATION SITES": PRINT
4560  PRINT : PRINT "-EACH VOLUN570  GOSUB 10000: GOTO 5500
4570  HTAB 2: PRINT "FOR OCCLUDED AND UNOCCLUDED (L/R) DATA-": PRINT : PRINT
4580  INVERSE : HTAB 7: PRINT "'C'-CORRECT / 'I'-INCORRECT": NORMAL
4590  GOSUB 9980: INPUT W$
4600  IF W$ (  ) "C" THEN  HOME : VTAB 12: HTAB 10: PRINT "RE-COMPLETE SECTION 1": GOSUB 10000: GOTO 10
4610  HOME : PRINT : PRINT "FOR EACH VOLUNTEER AT EACH TIME INTERVAL"
4620  HTAB 6: PRINT "YOU WILL BE ASKED TO TYPE IN:": PRINT : PRINT
4630  PRINT "-THE INITIAL OF THE OBSERVER": PRINT
4640  PRINT "-THE APPLICATION MODE (OCCLUDED OR NOT)": PRINT
4650  PRINT "-THE NUMBER OF THE VOLUNTEER": PRINT
4660  PRINT "-THE DATA RECORDING INTERVAL": PRINT
4670  PRINT "-THE APPLICATION PATTERN USED": PRINT
4680  PRINT "-THE ACTUAL BLANCHING SCORES": PRINT
4690  PRINT "-THE COMPARISONS BETWEEN SITES": PRINT : PRINT
4700  HTAB 5: INVERSE : PRINT "'C' TO CONTINUE WITH AN EXAMPLE": NORMAL : GOSUB 9980
4710  INPUT W$: IF W$ (  ) "C" THEN  GOTO 10
4720  HOME : HTAB 15: INVERSE : FLASH : PRINT "EXAMPLE": NORMAL : PRINT
4730  INVERSE : PRINT "OBSERVER'S INITIAL";: NORMAL : PRINT " ( ";
4740  FOR A = 1 TO O1: PRINT O$(A);: PRINT " ";: NEXT A
4750  PRINT ")   :";O$(1)
4760  PRINT : INVERSE : PRINT "OCCLUDED OR UNOCCLUDED";: NORMAL : PRINT " (O OR U):U"
4770  PRINT : INVERSE : PRINT "VOLUNTEER'S NUMBER";: NORMAL : PRINT " ( 1 TO ";V1;" ) :";V1
4780  PRINT : INVERSE : PRINT "DATA RECORDING PERIOD";: NORMAL : PRINT " (1 TO ";T1;"):";(T1 - 1)
4790  PRINT : INVERSE : PRINT "APPLIC. PATTERN USED";: NORMAL : PRINT " (1 TO ";P1;")  :";(P1 - 1)
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4800  PRINT : INVERSE : PRINT "ACTUAL BLANCHING SCORES";: NORMAL : PRINT " (0 TO 4)"
4810  PRINT : PRINT " ";: INVERSE : PRINT "--12 SITES--": NORMAL
4820  PRINT " 1213012...ETC."
4830  PRINT : INVERSE : PRINT "COMPARISONS";: NORMAL : PRINT " ( ( ) 0 OR = )": PRINT
4840  PRINT " ";: INVERSE : PRINT "----10----": NORMAL
4850  PRINT " (()==000()...ETC.": PRINT : GOSUB 9980
4860  HTAB 5: INVERSE : PRINT "'C' TO CONTINUE WITH DATA INPUT": NORMAL
4870  INPUT W$: IF W$ ( ) "C" THEN  GOTO 10
4880  GOTO 3150
4890  REM ################################################## 3
4900  HOME : HTAB 1: PRINT "THIS SECTION DEALS WITH THE STATISTICAL": PRINT
4910  HTAB 3: PRINT "ANALYSIS OF BLANCHING DATA ALREADY": PRINT
4920  HTAB 8: PRINT "STORED ON THE DATA DISK": PRINT : PRINT
4930  VTAB 9: HTAB 3: PRINT "IF ALL THE BLANCHING DATA FOR EACH"
4940  HTAB 6: PRINT "---"
4950  HTAB 2: PRINT "OBSERVER, EACH VOLUNTEER & EACH TIME": PRINT
4960  PRINT "INTERVAL HAS NOT BEEN STORED ON THE DATA"
4970  HTAB 2: PRINT "DISK IN DRIVE 2, RETURN AND COMPLETE": PRINT
4980  HTAB 3: PRINT "SECTION 2 OF THIS PROGRAMME FIRST": PRINT
4990  GOSUB 9980: GOSUB 9940
5000  INPUT W$: IF W$ ( ) "C" THEN  GOTO 10
5010  HOME : VTAB 4: HTAB 6: PRINT "NB. THIS SECTION REQUIRES THE"
5020  VTAB 6: HTAB 9: PRINT "PRINTER TO BE CONNECTED"
5030  VTAB 10: HTAB 1: PRINT "INSERT DATA DISK INTO DRIVE 2 AND CLOSE"
5040  VTAB 12: HTAB 12: PRINT "DISK DRIVE DOOR"
5050  VTAB 18: INVERSE : HTAB 12: PRINT "'C' TO CONTINUE"
5060  VTAB 20: PRINT "'R' TO SHUT-DOWN FOR PRINTER CONNECTION": NORMAL
5070  GOSUB 9980: INPUT W$: IF W$ ( ) "C" THEN  GOTO 10
5080  HOME : VTAB 12: HTAB 18: PRINT "WAIT"
5090  PRINT Z$;"OPEN MISC,D2"
5100  PRINT Z$;"READ MISC"
5110  INPUT V1,T1,H1$,P1,P2,C1,R1,O1
5120  PRINT Z$;"CLOSE"
5130  HOME : VTAB 6: HTAB 9: PRINT "THIS DISK IS LABELLED:"
5140  VTAB 12: HTAB 6: PRINT H1$
5150  VTAB 18: HTAB 12: INVERSE : PRINT "'C' TO CONTINUE": PRINT : GOSUB 9980
5160  HTAB 8: PRINT "'R' TO REPLACE DATA DISK": NORMAL
5170  INPUT W$: IF W$ = "R" THEN  GOTO 5010
5180  IF W$ ( ) "C" THEN  GOTO 10
5190  HOME : VTAB 12: HTAB 18: PRINT "WAIT"
5200  DIM O$(O1): DIM PSITE$(P1): DIM T$(T1): DIM PREP$(R1): DIM C$(C1): DIM OBS$(O1): DIM VOL$(V1)
5210  DIM Z(R1): DIM N(R1): DIM W(R1): DIM H(R1): DIM F(R1): DIM R(C1): DIM Q(C1): DIM AS(T1): DIM PTPS(T1)
5220  PRINT Z$;"OPEN OBSERVERS,D2"
5230  PRINT Z$;"READ OBSERVERS"
5240  INPUT O1
5250  FOR A = 1 TO O1
5260  INPUT O$(A): NEXT
5270  PRINT Z$;"CLOSE"
5280  HOME : VTAB 1: HTAB 2: PRINT "THE DATA CAN BE ANALYSED IN A NUMBER": PRINT
5290  HTAB 15: PRINT "OF WAYS:": PRINT : PRINT
5300  PRINT "ACCORDING TO OBSERVERS-INCLUDING ALL": PRINT
5310  HTAB 23: PRINT "-USING 1 OBSERVER": PRINT
5320  HTAB 23: PRINT "-USING 2 OBSERVERS"
5330  HTAB 23: PRINT "-ETC.": PRINT : PRINT
5340  PRINT "ACCORDING TO VOLUNTEERS-USING THE DATA": PRINT
5350  HTAB 25: PRINT "FROM ANY NUMBER": PRINT
5360  HTAB 25: PRINT "OF THESE": PRINT
5370  PRINT "OR ANY COMBINATION OF THE ABOVE": PRINT
5380  HTAB 12: INVERSE : PRINT "'C' TO CONTINUE": NORMAL : GOSUB 9980
5390  INPUT W$: IF W$ ( ) "C" THEN  GOTO 10
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5400  HOME : VTAB 4: HTAB 16: INVERSE : PRINT "OBSERVERS": NORMAL
5410  VTAB 8: PRINT "TYPE IN THE NUMBER OF OBSERVERS YOU WISH"
5420  HTAB 5: PRINT "TO PARTICIPATE IN THE STATISTICS": PRINT : GOSUB 9980
5430  HTAB 16: INVERSE : PRINT "(1  -  ";01;")": NORMAL : PRINT
5440  INPUT 02: IF 02 = 01 THEN  GOTO 5660: REM  #/ OBSERVERS IN STATS
5450  IF 02 ) 01 THEN  PRINT : PRINT "NUMBER TOO LARGE -OPTIONS:": GOSUB 10000: PRINT : GOTO 5430
5460  IF 02 ( 1 THEN  GOSUB 10000: GOTO 5400
5470  PRINT : HTAB 3: PRINT "TYPE IN THE INITIAL OF THE OBSERVER": PRINT
5480  HTAB 8: PRINT "YOU WISH TO PARTICIPATE": PRINT
5490  FOR A = 1 TO 02: GOSUB 9980
5500  INVERSE : PRINT "OBSERVER ";A;: NORMAL : PRINT ":";
5510  INPUT OBS$(A): PRINT : REM  INITIALS OF OBSERVERS IN STATS
5520  FOR B = 1 TO 01
5530  IF OBS$(A) = O$(B) THEN  GOTO 5580
5540  NEXT B
5550  PRINT "INVALID INITIAL: OPTIONS ";
5560  FOR B = 1 TO 01: PRINT O$(B);" ";: NEXT B: PRINT : PRINT
5570  GOSUB 10000: GOTO 5500
5580  NEXT A
5590  IF 02 = 1 THEN  GOTO 5690
5600  FOR A = 1 TO (02 - 1)
5610  FOR B = (A + 1) TO 02
5620  IF OBS$(A) = OBS$(B) THEN  PRINT "INVALID INITIAL: REPITITION": GOSUB 10000: PRINT : GOTO 5470
5630  NEXT B
5640  NEXT A
5650  GOTO 5690
5660  FOR A = 1 TO 01
5670  LET OBS$(A) = O$(A)
5680  NEXT A
5690  HOME : VTAB 3: HTAB 15: INVERSE : PRINT "VOLUNTEERS": NORMAL
5700  VTAB 6: HTAB 2: PRINT "TYPE IN THE NUMBER OF VOLUNTEERS YOU": PRINT
5710  HTAB 4: PRINT "WOULD LIKE TO PARTICIPATE IN THE": PRINT
5720  HTAB 16: PRINT "STATISTICS": PRINT
5730  HTAB 16: INVERSE : PRINT "( 1 - ";V1;" )": PRINT : NORMAL : GOSUB 9980
5740  INPUT V3: IF V3 ) V1 THEN  PRINT : PRINT "NUMBER TOO LARGE: OPTIONS 1 TO ";V1: PRINT : GOSUB 10000
5750  IF V3 ( 1 THEN  GOSUB 10000: GOTO 5690
5760  IF V3 = V1 THEN  GOTO 5930
5770  PRINT : PRINT : PRINT
5780  HTAB 1: PRINT "FOR EACH VOLUNTEER TO BE INCLUDED TYPE": PRINT
5790  HTAB 2: PRINT "IN HIS/HER VOLUNTEERS NUMBER AS SHOWN": PRINT
5800  HTAB 6: PRINT "ON THE BLANCHING SCORING CARD": PRINT
5810  FOR A = 1 TO V3
5820  INVERSE : PRINT "VOLUNTEER ";A;: NORMAL : PRINT ":";: GOSUB 9980
5830  INPUT VOL$(A): PRINT : REM  #'S OF VOLUNTEERS IN STATS
5840  IF  VAL (VOL$(A)) ) V1 THEN  PRINT "NUMBER TOO LARGE: OPTIONS 1 TO ";V1: PRINT : GOSUB 10000: GOTO 5820
5850  IF  VAL (VOL$(A)) ( 1 THEN  GOSUB 10000: PRINT : PRINT : GOTO 5820
5860  NEXT A: PRINT
5870  FOR A = 1 TO (V3 - 1)
5880  FOR B = (A + 1) TO V3
5890  IF VOL$(A) = VOL$(B) THEN  PRINT : PRINT : PRINT "REPITITION IN NUMBERS": GOSUB 10000: GOTO 5780
5900  NEXT B
5910  NEXT A
5920  GOTO 5960
5930  FOR A = 1 TO V3
5940  LET VOL$(A) =  STR$ (A)
5950  NEXT A
5960  HOME : HTAB 12: INVERSE : FLASH : PRINT "CHECK YOUR ENTRY": NORMAL : PRINT : GOSUB 9980
5970  HTAB 10: INVERSE : PRINT "DATA TO BE INCLUDED:": PRINT
5980  HTAB 8: PRINT "OBSERVERS";: NORMAL : PRINT "     ";: INVERSE : PRINT "VOLUNTEERS": NORMAL : PRINT
5990  VTAB 7: FOR A = 1 TO 02: HTAB 12: PRINT OBS$(A): PRINT : NEXT A
6000  VTAB 7: FOR A = 1 TO V3: HTAB 26: PRINT VOL$(A): NEXT A
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6010  VTAB 21: HTAB 9: INVERSE : PRINT "'C' TO CONTINUE": PRINT
6020  HTAB 9: PRINT "'R' TO RE-ENTER": NORMAL
6030  INPUT W$: IF W$ = "R" THEN  GOTO 5400
6040  IF W$ (  ) "C" THEN  GOTO 10
6050  HOME : VTAB 8: HTAB 2: PRINT "DO YOU WISH THE DATA FROM THE OCCLUDED": PRINT
6060  PRINT "AND UNOCCLUDED (LEFT & RIGHT) ARMS TO BE"
6070  HTAB 3: PRINT "EVALUATED SEPARATELY OR TOGETHER"
6080  VTAB 15: HTAB 8: FLASH : PRINT "** SEPARATE / TOGETHER **": NORMAL : PRINT : GOSUB 9980
6090  INPUT SEPT$: VTAB 19: INVERSE
6100  IF SEPT$ = "TOGETHER" THEN  PRINT "COLLECTIVE DATA ANALYSIS FROM L & R ARMS": GOTO 6120
6110  HTAB 2: PRINT "SEPARATE ANALYSIS OF
6120  FOR BEEP = 1 TO 40:SOUND =  PEEK (S): NEXT
6130  VTAB 21: HTAB 6: PRINT "'C' TO CONTINUE WITH AMALYSIS": PRINT
6140  HTAB 9: PRINT "'R' TO RE-ENTER OPTION": NORMAL
6150  INPUT W$: IF W$ = "R" THEN  GOTO 6050
6160  IF W$ (  ) "C" THEN  GOTO 10
6170  HOME : VTAB 12: HTAB 6: PRINT "CHECKING DATA - PLEASE WAIT"
6180  FOR BEEP = 1 TO 40:SOUND =  PEEK (S): NEXT BEEP
6190  FOR BEEP = 1 TO 40:SOUND =  PEEK (S) -  PEEK (S): NEXT BEEP
6200  FOR BEEP = 1 TO 40:SOUND =  PEEK (S): NEXT BEEP
6210  PRINT Z$;"OPEN PATTERNS,D2"
6220  PRINT Z$;"READ PATTERNS"
6230  INPUT P1
6240  INPUT P2
6250  FOR A = 1 TO P1
6260  INPUT PSITE$(A)
6270  NEXT
6280  PRINT Z$;"CLOSE"
6290  LET R1$ = "ABCDEFGHIJKL"
6300  LET R1$ =  MID$ (R1$,1,R1)
6310  FOR A = 1 TO 02
6320  FOR B = 1 TO V3
6330  PRINT Z$;"OPEN";OBS$(A);VOL$(B);",L";(P2 + C1 + 7);",D2"
6340  PRINT Z$;"READ";OBS$(A);VOL$(B);",R";(T1 + 1)
6350  INPUT NUMBERS$(1)
6360  PRINT Z$;"READ";OBS$(A);VOL$(B);",R";((T1 + 1) * 2)
6370  INPUT NUMBERS$(2)
6380  PRINT Z$;"CLOSE"
6390  LET OCC$ = "0"
6400  FOR E = 1 TO 2
6410  FOR D = 1 TO  LEN (NUMBERS$(E))
6420  IF  MID$ (NUMBERS$(E),D,1) = "X" THEN  GOTO 6730
6430  NEXT D
6440  LET OCC$ = "U": NEXT E
6450  PRINT Z$;"OPEN";OBS$(A);VOL$(B);",L";(P2 + C1 + 7);",D2"
6460  FOR C = 1 TO T1
6470  PRINT Z$;"READ";OBS$(A);VOL$(B);",R";C
6480  INPUT NUN(C)
6490  NEXT
6500  FOR D = (T1 + 2) TO ((T1 * 2) + 1)
6510  PRINT Z$;"READ";OBS$(A);VOL$(B);",R";D
6520  INPUT NUN(D)
6530  NEXT
6540  PRINT Z$;"CLOSE"
6550  LET OCC$ = "0"
6560  FOR C = 2 TO T1
6570  IF NUN(1) (  ) NUN(C) THEN  GOTO 6660
6580  NEXT C
6590  LET OCC$ = "U"
6600  FOR D = (T1 + 3) TO ((T1 * 2) + 1)
6610  IF NUN(T1 + 2) (  ) NUN(D) THEN  GOTO 6660
```

```
6620  NEXT D
6630  NEXT B
6640  NEXT A
6650  GOTO 6800
6660  HOME : HTAB 7: FLASH : PRINT "APPLICATION PATTERN ERROR": NORMAL : GOSUB 10000
6670  VTAB 3: PRINT "OBSERVER: ";OBS$(A); SPC( 2);"OCC/UNOCC: ";OCC$; SPC( 2);"VOLUNTEER: "B
6680  VTAB 5: HTAB 4: PRINT "TIME INTERVAL    PATTERN RECORDED"
6690  IF OCC$ = "O" THEN  FOR C = 1 TO T1: HTAB 9: PRINT C;: HTAB 30: PRINT MUN(C): NEXT C: GOTO 6710
6700  IF OCC$ = "U" THEN  FOR D = 1 TO T1: HTAB 9: PRINT D;: HTAB 30: PRINT MUN(T1 + 1 + D): NEXT D
6710  VTAB 23: INVERSE : HTAB 7: PRINT "'R' TO RETURN TO SECTION 2": NORMAL
6720  INPUT W$: GOTO 10
6730  PRINT : HTAB 12: INVERSE : FLASH : PRINT "INCOMPLETE DATA": NORMAL : PRINT : GOSUB 10000
6740  PRINT "OBSERVER: ";OBS$(A): PRINT : PRINT "VOLUNTEER: ";VOL$(B): PRINT : PRINT "OCC / UNOCC: ";OCC$
6750  PRINT : PRINT "'X' DENOTES TIME INTERVAL WITH NO DATA": PRINT
6760  INVERSE : HTAB 2: PRINT "'C'-TO TYPE NEW OBSERVERS / VOLUNTEERS": HTAB 9: PRINT "FOR STATISTICAL ANALYSIS"
6770  PRINT "'R'-RETURN TO PART 2 FOR DATA COMPLETION": NORMAL : PRINT
6780  INPUT W$: IF W$ = "C" THEN  GOTO 5400
6790  IF W$ (  ) "C" THEN  GOTO 10
6800  HOME : VTAB 12: HTAB 10: PRINT "ANALYSIS IN PROGRESS": PRINT : HTAB 6
6810  FOR BEEP = 1 TO 40:SOUND =  PEEK (S): NEXT BEEP
6820  FOR BEEP = 1 TO 40:SOUND =  PEEK (S) -  PEEK (S): NEXT BEEP
6830  FOR BEEP = 1 TO 40:SOUND =  PEEK (S): NEXT BEEP
6840  VTAB 22: HTAB 3: PRINT "((THE SCREEN CAN BE SWITCHED OFF))"
6850  IF SEPT$ = "TOGETHER" THEN  PRINT Z$;"BLOAD CHAIN,A520,D1": CALL 520"TOGETHER
6860  IF C1 = 0 THEN  GOTO 7140
6870  FOR A = 1 TO P1
6880  PRINT Z$;"OPEN COMPS";A;",L10,D2"
6890  FOR K = 1 TO C1
6900  PRINT Z$;"READ COMPS";A;",R";K
6910  INPUT COM$(A,K)
6920  NEXT K
6930  PRINT Z$;"CLOSE"
6940  NEXT A
6950  DIM PAR$(C1 * 2)
6960  LET A = 1
6970  FOR M = 1 TO C1
6980  FOR L = 1 TO  LEN (COM$(1,M))
6990  IF  MID$ (COM$(1,M),L,1) = "/" THEN  GOTO 7010
7000  NEXT L
7010  LET WUN$ =  MID$ (PSITE$(1),( VAL ( LEFT$ (COM$(1,M),(L - 1)))),1)
7020  LET TWO$ =  MID$ (PSITE$(1),( VAL ( RIGHT$ (COM$(1,M),( LEN (COM$(1,M)) - L)))),1)
7030  LET OCC$ = "O"
7040  LET PAR$(A) = OCC$ + WUN$ + TWO$
7050  LET OCC$ = "U"
7060  LET PAR$(A + 1) = OCC$ + WUN$ + TWO$
7070  IF A = 1 THEN  GOTO 7110
7080  FOR B = (A - 2) TO 1 STEP  - 2
7090  IF PAR$(A) = PAR$(B) THEN  GOTO 7120
7100  NEXT B
7110  LET A = A + 2
7120  NEXT M
7130  LET FIN = A - 1: REM   0/ COMPARISON FILES: OAB, UAB ETC.
7140  LET OCC$ = "O"
7150  FOR C = 1 TO T1: REM  TIME INTERVAL
7160  IF OCC$ = "U" THEN  FOR C = (T1 + 2) TO ((T1 * 2) + 1)
7170  FOR E = 1 TO R1
7180  LET Z(E) = 0: LET N(E) = 0: LET W(E) = 0: LET H(E) = 0: LET F(E) = 0
7190  NEXT E: IF C1 = 0 THEN  GOTO 7230
7200  FOR E = 1 TO FIN
7210  LET R(E) = 0: LET Q(E) = 0
7220  NEXT E
```

```
7230  FOR A = 1 TO 02: REM  OBSERVERS
7240  FOR B = 1 TO V3: REM  VOLUNTEERS
7250  PRINT Z$;"OPEN";OBS$(A);VOL$(B);",L";(P2 + C1 + 7);",D2"
7260  PRINT Z$;"READ";OBS$(A);VOL$(B);",R";C
7270  INPUT P3: INPUT BLANCH$: INPUT COMPAR$
7280  PRINT Z$;"CLOSE"
7290  FOR D = 1 TO P2: REM  #/ SITES
7300  FOR E = 1 TO R1
7310  IF  MID$ (PSITE$(P3),D,1) <  > MID$ (R1$,E,1) THEN  NEXT E
7320  IF  MID$ (BLANCH$,D,1) = "0" THEN  LET Z(E) = Z(E) + 1
7330  IF  MID$ (BLANCH$,D,1) = "1" THEN  LET N(E) = N(E) + 1
7340  IF  MID$ (BLANCH$,D,1) = "2" THEN  LET W(E) = W(E) + 1
7350  IF  MID$ (BLANCH$,D,1) = "3" THEN  LET H(E) = H(E) + 1
7360  IF  MID$ (BLANCH$,D,1) = "4" THEN  LET F(E) = F(E) + 1
7370  NEXT D
7380  IF C1 = 0 THEN  GOTO 7550
7390  FOR J = 1 TO C1: REM  #/ COMPARISONS
7400  IF  MID$ (COMPAR$,J,1) = ")" THEN  GOTO 7440
7410  IF  MID$ (COMPAR$,J,1) = "(" THEN  GOTO 7440
7420  NEXT J
7430  GOTO 7550
7440  FOR L = 1 TO  LEN (COM$(P3,J))
7450  IF  MID$ (COM$(P3,J),L,1) = "/" THEN  GOTO 7470
7460  NEXT L
7470  LET WUN$ =  MID$ (PSITE$(P3),( VAL ( LEFT$ (COM$(P3,J),(L - 1)))),1)
7480  LET TWO$ =  MID$ (PSITE$(P3),( VAL ( RIGHT$ (COM$(P3,J),( LEN (COM$(P3,J)) - L)))),1)
7490  FOR K = 1 TO FIN
7500  IF PAR$(K) = OCC$ + WUN$ + TWO$ THEN  GOTO 7520
7510  NEXT K
7520  IF  MID$ (COMPAR$,J,1) = ")" THEN  LET R(K) = R(K) + 1
7530  IF  MID$ (COMPAR$,J,1) = "(" THEN  LET Q(K) = Q(K) + 1
7540  NEXT J
7550  NEXT B
7560  NEXT A
7570  IF C1 = 0 THEN  GOTO 7660
7580  IF OCC$ = "0" THEN  FOR K = 1 TO (FIN - 1) STEP 2
7590  IF OCC$ = "U" THEN  FOR K = 2 TO FIN STEP 2
7600  PRINT Z$;"OPEN";PAR$(K);",L10,D2"
7610  IF OCC$ = "0" THEN  PRINT Z$;"WRITE";PAR$(K);",R";C
7620  IF OCC$ = "U" THEN  PRINT Z$;"WRITE";PAR$(K);",R";(C - (T1 + 1))
7630  PRINT R(K): PRINT Q(K): REM  #/ )'S & ('S
7640  PRINT Z$;"CLOSE"
7650  NEXT
7660  PRINT Z$;"MON,C,I,O"
7670  IF OCC$ = "0" THEN  PRINT Z$;"OPEN";OCC$;C;",L30,D1"
7680  IF OCC$ = "U" THEN  PRINT Z$;"OPEN";OCC$;(C - (T1 + 1));",L30,D1"
7690  FOR G = 1 TO R1
7700  IF OCC$ = "0" THEN  PRINT Z$;"WRITE";OCC$;C;",R";G
7710  IF OCC$ = "U" THEN  PRINT Z$;"WRITE";OCC$;(C - (T1 + 1));",R";G
7720  PRINT Z(G): PRINT N(G): PRINT W(G): PRINT H(G): PRINT F(G)
7730  NEXT
7740  PRINT Z$;"CLOSE"
7750  PRINT Z$;"NOMON,C,I,O"
7760  NEXT C
7770  IF OCC$ = "U" GOTO 7790
7780  LET OCC$ = "U": GOTO 7160
7790  HOME : VTAB 12: HTAB 10: PRINT "CALCULATING %TPS...."
7800  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
7810  FOR BEEP = 1 TO 40:SOUND =  PEEK (S) -  PEEK (S): NEXT BEEP
7820  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
7830  LET TPS = 4 * 02 * (P2 / R1) * V3: REM  TOTAL POSSIBLE SCORE
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7835  GOSUB 8320
7840  LET OCC$ = "O"
7850  PRINT Z$;"PR#1": PRINT : PRINT "APPLICATION MODE: ";OCC$
7855  FOR A = 1 TO T1
7860  PRINT Z$;"OPEN";OCC$;A;",L30,D1"
7870  FOR B = 1 TO R1
7880  PRINT Z$;"READ";OCC$;A;",R";B
7890  INPUT Z(B): INPUT N(B): INPUT W(B): INPUT H(B): INPUT F(B)
7900  NEXT
7910  PRINT Z$;"CLOSE"
7920  FOR B = 1 TO R1
7924  PRINT Z$;"PR#1": PRINT "INTERVAL: ";A;"  PREP: "; MID$ (R1$,B,1)
7930  LET AS(B) = N(B) + (W(B) * 2) + (H(B) * 3) + (F(B) * 4): REM  AS= ACTUAL SCORE
7940  LET PTPS(B) = (AS(B) / TPS) * 100: REM   PTPS= % TPS
7950  NEXT B
7955  PRINT : PRINT Z$;"PR#O"
7960  FOR B = 1 TO R1
7970  PRINT Z$;"OPEN PREP";B;OCC$;",L30,D2"
7980  PRINT Z$;"WRITE PREP";B;OCC$;",R";A
7990  PRINT AS(B): PRINT PTPS(B)
8000  PRINT Z$;"CLOSE"
8010  NEXT B
8020  NEXT A
8030  IF OCC$ = "U" THEN  GOTO 8050
8040  LET OCC$ = "U": GOTO 7850
8050  HOME : VTAB 12: HTAB 10: PRINT "CALCULATING AUC....."
8060  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
8070  FOR BEEP = 1 TO 40:SOUND =  PEEK (S) -  PEEK (S): NEXT BEEP
8080  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
8090  PRINT Z$;"OPEN TIMES,L5,D2"
8100  FOR A = 1 TO T1
8110  PRINT Z$;"READ TIMES,R";A
8120  INPUT TM(A)
8130  NEXT
8140  PRINT Z$;"CLOSE"
8150  LET OCC$ = "O"
8160  FOR A = 1 TO R1
8170  PRINT Z$;"OPEN PREP";A;OCC$;",L30,D2"
8180  FOR B = 1 TO T1
8190  PRINT Z$;"READ PREP";A;OCC$;",R";B
8200  INPUT AS(B): INPUT PT7: PRINT "AUC = ";AUC(D)
8210  NEXT
8220  LET TM(0) = 0: LET PTPS(0) = 0: LET AUC = 0
8230  FOR B = 0 TO (T1 - 1)
8240  LET AUC = AUC + ((PTPS(B) + PTPS(B + 1)) / 2) * (TM(B + 1) - TM(B))
8250  NEXT B
8260  PRINT Z$;"WRITE PREP";A;OCC$;",R";(T1 + 1)
8270  PRINT AUC
8280  PRINT Z$;"CLOSE"
8290  NEXT A
8300  IF OCC$ = "U" THEN  GOTO 8478
8310  LET OCC$ = "U": GOTO 8160
8320  PRINT Z$;"OPEN PREPARATIONS,D2"
8330  PRINT Z$;"READ PREPARATIONS"
8340  FOR A = 1 TO R1
8350  INPUT PREP$(A)
8360  NEXT
8370  PRINT Z$;"CLOSE"
8380  PRINT Z$;"PR#1"
8390  HTAB 22: PRINT H1$
8400  HTAB 22: FOR A = 1 TO  LEN (H1$): PRINT "=";: NEXT A: PRINT : PRINT : PRINT
8410  PRINT "OBSERVERS USED IN STATS: ";
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8420  FOR A = 1 TO 02: PRINT OBS$(A);"  ";: NEXT A: PRINT : PRINT
8430  PRINT "VOLUNTEERS USED IN STATS: ";
8440  FOR A = 1 TO V3: PRINT VOL$(A);" ";: NEXT A: PRINT : PRINT
8450  PRINT "PREPARATIONS USED IN THIS TRIAL:": PRINT
8460  FOR A = 1 TO R1
8470  HTAB 4: PRINT "PREP "; MID$ (R1$,A,1);" = ";PREP$(A): NEXT A: PRINT
8476  PRINT Z$;"PR#0": RETURN
8478  PRINT Z$;"PR#1": PRINT
8480  PRINT "GRADED RESPONSE STATISTICS (CHI-SQUARED VALUES: 95% LEVEL OF SIG.)"
8490  PRINT "-------------------------------------------------------------------": PRINT
8500  PRINT "(CHI-SQUARED VALUES ) 9.488 ARE SIGNIFICANT)": PRINT
8510  PRINT Z$;"PR#0"
8520  HOME : VTAB 12: HTAB 9: PRINT "GRADED RESPONSE STATS...."
8530  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
8540  FOR BEEP = 1 TO 40:SOUND =  PEEK (S) -  PEEK (S): NEXT BEEP
8550  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
8560  LET OCC$ = "O"
8570  FOR A = 1 TO T1
8580  PRINT Z$;"OPEN";OCC$;A;",L30,D1"
8590  FOR B = 1 TO R1
8600  PRINT Z$;"READ";OCC$;A;",R";B
8610  INPUT Z(B): INPUT M(B): INPUT W(B): INPUT H(B): INPUT F(B)
8620  NEXT
8630  PRINT Z$;"CLOSE"
8640  FOR B = 1 TO (R1 - 1)
8650  FOR C = (B + 1) TO R1
8660  LET U(1) = Z(B): LET U(2) = M(B): LET U(3) = W(B): LET U(4) = H(B): LET U(5) = F(B)
8670  LET X(1) = Z(C): LET X(2) = M(C): LET X(3) = W(C): LET X(4) = H(C): LET X(5) = F(C)
8680  GOSUB 8750
8690  NEXT C
8700  NEXT B
8710  NEXT A
8720  IF OCC$ = "U" THEN  GOTO 8950
8730  PRINT Z$;"PR#1": PRINT : PRINT Z$;"PR#0"
8740  LET OCC$ = "U": GOTO 8570
8750  LET COL(1) = 0: LET COL(2) = 0: LET CHI = 0
8760  FOR D = 1 TO 5
8770  LET COL(1) = COL(1) + U(D)
8780  LET COL(2) = COL(2) + X(D)
8790  NEXT D
8800  FOR D = 1 TO 5
8810  LET ROW(D) = U(D) + X(D)
8820  NEXT D
8830  LET TAL = COL(1) + COL(2)
8840  FOR D = 1 TO 5
8850  IF ((COL(1) * ROW(D)) / TAL) = 0 THEN  LET CHI = CHI + ((U(D) - 1 * 10 ^  - 20) ^ 2): GOTO 8870
8860  LET CHI = CHI + ((U(D) - ((COL(1) * ROW(D)) / TAL)) ^ 2) / ((COL(1) * ROW(D)) / TAL)
8870  IF ((COL(2) * ROW(D)) / TAL) = 0 THEN  LET CHI = CHI + ((X(D) - 1 * 10 ^  - 20) ^ 2): GOTO 8890
8880  LET CHI = CHI + ((X(D) - ((COL(2) * ROW(D)) / TAL)) ^ 2) / ((COL(2) * ROW(D)) / TAL)
8890  NEXT D
8910  PRINT Z$;"PR#1"
8920  PRINT "OCC/UNOCC: ";OCC$;"     INTERVAL: ";A;"     PREPS: "; MID$ (R1$,B,1);"/"; MID$ (R1$,C,1);
8930  PRINT Z$;"PR#0"
8940  RETURN
8950  IF C1 = 0 THEN  GOTO 9230
8960  PRINT Z$;"PR#1"
8970  PRINT : PRINT : PRINT "SITE COMPARISON STATISTICS (CHI-SQUARED VALUES: 95% LEVEL OF SIG.)
8980  PRINT "-------------------------------------------------------------------": PRINT
8990  PRINT "(CHI SQUARED VALUES ) 3.841 ARE SIGNIFICANT)": PRINT
9000  PRINT Z$;"PR#0"
9010  HOME : VTAB 12: HTAB 8: PRINT "SITE COMPARISON STATISTICS...."
9020  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
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9030  FOR BEEP = 1 TO 40:SOUND =  PEEK (S) -  PEEK (S): NEXT BEEP
9040  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
9050  FOR A = 1 TO FIN
9060  PRINT Z$:"OPEN";PAR$(A);",L10,D2"
9070  FOR B = 1 TO T1
9080  PRINT Z$;"READ";PAR$(A);",R";B
9090  INPUT R(B): INPUT Q(B)
9100  NEXT
9110  PRINT Z$;"CLOSE"
9120  FOR B = 1 TO T1
9130  LET TAL = R(B) + Q(B)
9140  IF TAL = 0 THEN  LET TAL = 1 * 10 ^  - 20
9150  LET CHI = (((R(B) - (TAL / 2)) ^ 2) / (TAL / 2)) + (((Q(B) - (TAL / 2)) ^ 2) / (TAL / 2))
9170  PRINT Z$;"PR#1"
9180  PRINT "OCC/UNOCC & PREPS: ";PAR$(A);"  INTERVAL: ";B;"  )'S: ";R(B);"  ('S: ";Q(B);"  CHI-SQ: ";CHI
9190  PRINT Z$;"PR#0"
9200  NEXT B
9210  PRINT Z$;"PR#1": PRINT : PRINT Z$;"PR#0"
9220  NEXT A
9230  HOME : VTAB 12: HTAB 11: PRINT "PRINTING RESULTS...."
9240  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
9250  FOR BEEP = 1 TO 40:SOUND =  PEEK (S) -  PEEK (S): NEXT BEEP
9260  FOR BEEP = 1 TO 80:SOUND =  PEEK (S): NEXT BEEP
9270  PRINT Z$;"OPEN TIMES,L5,D2"
9280  FOR A = 1 TO T1
9290  PRINT Z$;"READ TIMES,R";A
9300  INPUT T$(A)
9310  NEXT
9320  PRINT Z$;"CLOSE"
9330  PRINT Z$;"PR#1"
9340  PRINT : PRINT : PRINT "ACTUAL SCORE / % TOTAL POSSIBLE SCORE / AREA UNDER CURVE VALUES"
9350  PRINT "------------------------------------------------------------"
9360  PRINT Z$;"PR#0"
9370  LET OCC$ = "O"
9380  FOR A = 1 TO R1 STEP 2
9390  LET D = A + 1
9400  PRINT Z$;"OPEN PREP";A;OCC$;",L30,D2"
9410  FOR B = 1 TO T1
9420  PRINT Z$;"READ PREP";A;OCC$;",R";B
9430  INPUT AS(B): INPUT PTPS(B)
9440  NEXT
9450  PRINT Z$;"READ PREP";A;OCC$;",R"(T1 + 1)
9460  INPUT AUC(A)
9470  PRINT Z$;"CLOSE"
9480  IF D ) R1 THEN  LET D = D - 2: GOTO 9570
9490  PRINT Z$;"OPEN PREP";D;OCC$;",L30,D2"
9500  FOR B = 1 TO T1
9510  PRINT Z$;"READ PREP";D;OCC$;",R";B
9520  INPUT SA(B): INPUT SPTP(B)
9530  NEXT
9540  PRINT Z$;"READ PREP";D;OCC$;",R"(T1 + 1)
9550  INPUT AUC(D)
9560  PRINT Z$;"CLOSE"
9570  PRINT Z$;"PR#1"
9580  PRINT : PRINT : PRINT PREP$(A);: HTAB 40: PRINT PREP$(D)
9590  FOR C = 1 TO  LEN (PREP$(A)): PRINT "-";: NEXT C: HTAB 40: FOR C = 1 TO  LEN (PREP$(D)): PRINT "-";
9600  PRINT "OCC / UNOCC: ";OCC$;: HTAB 40: PRINT "OCC / UNOCC: ";OCC$: PRINT
9610  PRINT "HOURS";: HTAB 12: PRINT "AS";: HTAB 25: PRINT "%TPS";: HTAB 40: PRINT "HOURS";: POKE 36,51
9620  FOR B = 1 TO T1
9630  HTAB 3: PRINT TM(B);: HTAB 12: PRINT AS(B);: HTAB 22: PRINT PTPS(B);: POKE 36,43: PRINT TM(B);
9640  NEXT B
9650  HTAB 7: PRINT "AUC = ";AUC(A);: POKE 36,47: PRINT "AUC = ";AUC(D)
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9660  PRINT Z$;"PR#0"
9670  NEXT A
9680  IF OCC$ = "U" THEN  GOTO 9700
9690  LET OCC$ = "U": GOTO 9380
9700  HOME : VTAB 6: HTAB 9: PRINT "ANALYSIS COMPLETE...."
9710  FOR A = 1 TO 30
9720  FOR BEEP = 1 TO 20:SOUND =  PEEK (S): NEXT BEEP
9730  FOR PAUSE = 1 TO 20: NEXT PAUSE
9740  NEXT A: LET OCC$ = "0"
9750  VTAB 14: PRINT "*)PRESS '";: FLASH : PRINT "G";: NORMAL : PRINT "' FOR ROUGH GRAPH OF RESULTS(*"
9760  VTAB 16: PRINT "(*ONLY POSSIBLE WITH SILENTYPE PRINTER*)"
9770  VTAB 20: FLASH : PRINT ")) PRESS ANY OTHER KEY FOR MAIN MENU ((": NORMAL
9780  PRINT : INPUT W$: IF W$ (  ) "G" THEN  GOTO 10
9790  HOME : VTAB 12: HTAB 10: PRINT "DRAWING GRAPHS...."
9800  PRINT Z$"BLOAD CHAIN,A520,D1"
9810  CALL 520"GRAPH
9820  REM  ################################################## 4
9830  HOME : VTAB 4: HTAB 10: INVERSE : PRINT "* SHUT-DOWN PROCEDURE *": NORMAL
9840  VTAB 9: HTAB 4: PRINT "1. OPEN  BOTH  DISK  DRIVE  DOORS"
9850  VTAB 12: HTAB 4: PRINT "2. REMOVE BOTH DISKS & STORE AWAY"
9860  VTAB 15: HTAB 4: PRINT "3. SWITCH OFF COMPUTER AND SCREEN"
9870  VTAB 20: HTAB 14: FLASH : PRINT "** GOOD-BYE **": NORMAL
9880  GOSUB 9980
9890  FOR PAUSE = 1 TO 6000: NEXT
9900  FOR A = 1 TO 23
9910  CALL  - 922: FOR BEEP = 1 TO 5:SOUND =  PEEK (S): NEXT : FOR PAUSE = 1 TO 600: NEXT
9920  NEXT A
9930  HOME : NEW : END
9940  VTAB 20: INVERSE : HTAB 2: PRINT "PRESS 'C' TO CONTINUE OR 'R' TO RETURN"
9950  VTAB 22: HTAB 12: PRINT "TO THE MAIN MENU": NORMAL : RETURN
9960  VTAB 20: HTAB 8: INVERSE : PRINT "PRESS 'C' TO CONTINUE OR"
9970  VTAB 22: HTAB 12: PRINT "'R' TO RE-ENTER": NORMAL : RETURN
9980  FOR BEEP = 1 TO 7:SOUND =  PEEK (S): NEXT : RETURN
9990  HOME : VTAB 10: HTAB 4: PRINT "STORING INFORMATION-PLEASE WAIT": GOSUB 9980: RETURN
10000  FOR BEEP = 1 TO 200:SOUND =  PEEK (S) -  PEEK (S) +  PEEK (S) -  PEEK (S) +  PEEK (S): NEXT
```

REFERENCES

1. Higuchi,T. Physical chemical analysis of percutaneous absorption process from creams and ointments. J.Soc.Cosmet.Chem., 11, (1960), 85-97.

2. Barry,B.W. In "Dermatological Formulations", Barry,B.W. (Ed.), Marcel Dekker, New York, (1983), pp 55-67; 234-250.

3. Flynn,G.L., Yalkowsky,S.H. and Roseman,T.J. Mass transport phenomena and models: theoretical concepts. J.Pharm.Sci., 63, (1974), 479-510.

4. Guy,R.H. and Hadgraft,J. Mathematical models of percutaneous absorption. In "Percutaneous absorption: mechanisms--methodology--drug delivery", Bronaugh,R.L. and Maibach,H.I. (Eds.), Marcel Dekker, New York, (1985), 3-15.

5. Short,P.M., Abbs,E.T. and Rhodes,C.T. The effect of nonionic surfactants on the transport of testosterone across a cellulose acetate membrane. J.Pharm.Sci., 59, (1970), 995-998.

6. Rodgers,W.A., Buritz,R.S. and Alpert,D. The diffusion coefficient, solubility and permeability of helium in glass. J.Appl.Physiol., 25, (1954), 868-875.

7. Foreman,M.I. and Kelly,I. The diffusion of nandrolone through hydrated human cadaver skin. Br.J.Dermatol., 95, (1976), 265-270.

8. Guy,R.H., Hadgraf,J. and Maibach,H.I. A pharmacokinetic model for percutaneous absorption. Int.J.Pharm., 11, (1982), 119-129.

9. Guy,R.H. and Hadgraft,J. Physicochemical interpretation of the pharmacokinetics of percutaneous absorption. J.Pharmacokinet.Biopharm., 11, (1983),189-203.

10. Guy,R.H. and Hadgraft,J. Mathematical models in percutaneous absorption. In "Models in dermatology", 2, Maibach and Lowe, (Eds.), Karger, Basel, (1985), 170-177.

11. Hadgraft,J. Absorption of materialls by or through the living skin. Int.J.Cosmet.Sci., 7, (1985), 103-115.

12. Guy,R.H., Carlstrom,E.M., Bucks,D.A.W., Hinz,R.S. and Maibach,H.I. Percutaneous penetration of nicotinates: in vivo and in vitro measurements. J.Pharm.Sci., 75, (1986), 968-972.

13. Barry,B.W. and El Eini,D.I.D. Influence of non-ionic surfactants on permeation of hydrocortisone, dexamethasone, testosterone and progesterone across cellulose acetate membrane. J.Pharm.Pharmacol., 28, (1976), 219-227.

14. Poulsen,B.J., Young,E., Coquilla,V. and Katz,M. Effect of topical vehicle composition on the in vitro release of fluocinolone acetonide and its acetate ester. J.Pharm.Sci., 57, (1968), 928-933.

15. Bronaugh,R.L. and Stewart,R.F. Methods for in vitro percutaneous absorption studies V: permeation through damaged skin. J.Pharm.Sci., 74, (1985), 1962-1066.

16. Tojo,K., Chiang,C.C. and Chien,Y.W. Influence of donor solution upon skin permeation of drugs. J.Chem.Eng.(Japan), 19, (1986), 153-155.

17. Berenson,G.S. and Burch,G.E. Studies of diffusion of water through dead human skin: the effect of different environmental states and of chemical alterations of the epidermis. Am.J.Trop.Med.Hyg., 31, (1951), 842-853.

18. Potts,R.O. Stratum corneum hydration: experimental techniques and interpretation of results. J.Soc.Cosmet.Chem., 37, (1986), 9-33.

19. Dugard,P.H. and Scott,R.C. A method of predicting percutaneous absorption rates from vehicle to vehicle: an experimental assessment. Int.J.Pharm., 28, (1986), 219-227.

20. Harrison,S.M., Barry,B.W. and Dugard,P.H. Factors controlling liquid and vapour diffusion through human skin for the model compound benzyl alcohol. J.Pharm.Pharmacol., 35 (Suppl.), (1983), 31P.

21. Blank,I.H. Penetration of low-molecular-weight alcohols into the skin. J.Invest.Dermatol., 43, (1964), 415-420.

22. Blank,I.H. and Scheuplein,R.J. Transport into and within the skin. Br.J.Dermatol., 81, (1969), 4-10.

23. Onken,H.D. and Moyer,C.A. The water barrier in human epidermis. Arch.Dermatol., 87, (1963), 584-590.

24. Hadgraft,J., Walters,K.A. and Wotton,P.K. Facilitated percutaneous absorption: a comparison and evaluation of two in vitro models. Int.J.Pharm., 32, (1986), 257-263.

25. Coldman,M.F., Poulsen,B.J. and Higuchi,T. Enhancement of percutaneous absorption by the use off volatile:nonvolatile systems as vehicles. J.Pharm.Sci., 58, (1969), 1098-1102.

26. Akhter,S.A., Bennett,S.L., Waller,I.L. and Barry,B.W. An automated diffusion apparatus for studying skin penetration. Int.J.Pharm., 21, (1984), 17-26.

27. Bennett,S.L. and Barry,B.W. A comparison of the permeability of abdominal and male pattern baldness scalp skin. J.Pharm.Pharmacol. (Suppl.), 35, (1983), 32P.

28. Rochefort,A., Druot,P., Leduc,M., Vassalet,R. and Agache,P. A new technique for the evaluation of cosmetics effect on mechanical properties of stratum corneum and epidermis in vitro. Int.J.Cosmet.Sci., 8, (1986), 27-36.

29. Behl,C.R., Flynn,G.L., Kurihara,T., Harper,N., Smith,W. Higuchi,W.I., Ho,N.F.H. and Pierson,C.L. Hydration and percutaneous absorption:I. Influence of hydration on alkanol permeation through hairless mouse skin. J.Invest.Dermatol., 75, (1980), 346-352.

30. Scheuplein,R. In "The physiology and pathophysiology of the skin", 5, Jarrett,A.(Ed.), Academic Press, New York, (1978), 1692-1730.

31. Beastall,J., Guy,R.H., Hadgraft,J. and Wilding,I. The influence of urea on percutaneous absorption. Pharm.Res., 3, (1986), 294-297.

32. Parikh,N.H., Babar,A. and Plakogiannis,F.M. Medicament release from ointment bases: II. Testosterone: in vitro release and effects of additives on its release. Drug Dev.Ind.Pharm., 12, (1986), 2493-2509.

33. Foreman,M.I. Stratum corneum hydration; consequences for skin permeation experiments. Drug Dev.Ind.Pharm., 12, (1986), 461-463.

34. Valia,K.H. and Chien,Y.W. Effect of stratum corneum hydration. How serious is it? Drug Dev.Ind.Pharm., 12, (1986), 465-469.

35. AstleyJ.P. and Levine,M. Effect of dimethyl sulphoxide on permeability of human skin in vitro. J.Pharm.Sci., 65, (1976), 210-215.

36. Southwell,D. and Barry,B.W. The accelerant activity of 2-pyrrolidone in human stratum corneum, steady-state diffusion of model penetrants, methanol and N-octanol. J.Pharm.Pharmacol., 33 (Suppl.), (1981), 1P.

37. Sheth,N.V., Freeman,D.J., Higuchi,W.I. and Spruance,S.L. The influence of azone, propylene glycol and polyethylene glycol on in vitro skin penetration of trifluorothymidine. Int.J.Pharm., 28, (1986), 201-209.

38. Muktadir,A., Babar,A., Cutie,A.J. and Plakogiannis,F.M. Medicament release from ointment bases: III. Ibuprofen: In vitro release and in vivo absorption in rabbits. Drug Dev.Ind.Pharm., 12, (1986), 2521-2540.

39. Mirejovsky,D. and Takruri,H. Dermal penetration enhancement profile of hexamethylenelauramide and its homologues: in vitro versus in vivo behaviour of enhancers in the penetration of hydrocortisone. J.Pharm.Sci., 75, (1986), 1089-1093.

40. Julian,T.N. and Zentner,G.M. Ultrasonically mediated solute permeation through polymer barriers. J.Pharm.Pharmacol., 38, (1986), 871-877.

41. Harper Bellantone,N., Rim,S., Francoeur,M.L. and Rasadi,B. Enhanced percutaneous absorption via iontophoresis I. Evaluation of an in vitro system and transport of model compounds. Int.J.Pharm., 30, (1986). 63-72.

42. Keshary,P.R. and Chien,Y.W. Mechanisms of controlled nitroglycerin administration (I): development of a finite-dosing skin permeation system. Drug Dev.Ind.Pharm., 10, (1984), 883-913.

43. Frank-Kamenetskii,D.A. In "Diffusion and heat transfer in chemical kinetics". Plenum Press, New York, (1969).

44. Stehle,R.G. and Higuchi,W.I. In vitro model for transport of solutes in three-phase system II: experimental considerations. J.Pharm.Sci., 61, (1972), 1931-1935.

45. Stehle,R.G. and Higuchi,W.I. In vitro model for transport of solutes in three phase syatem I. Theoretical principles. J.Pharm.Sci., 61, (1972), 1922-1930.

46. Flynn,G.L. and Yalkowsky,S.H. Correlation and prediction of mass transport across membranes I: Influence of alkyl chain length in flux-determining properties of barrier and diffusant. J.Pharm.Sci., 61, (1972). 838-852.

47. Hunke,W.A. and Matheson,L.E. Mass transport properties of co(polyether)polyurethane membranes II: Permeability and sorption characteristics. J.Pharm.Sci., 70, (1981), 1313-1318.

48. Marzulli,F.N., Brown,D.W.C. and Maibach,H.I. Techniques for studying skin penetration. Toxicol.Appl.Pharmacol., Suppl.3, (1969), 79-83.

49. Franz,T.J. Percutaneous absorption. On the relevance of in vitro data. J.Invest.Dermatol., 64, (1975), 190-195.

50. Franz,T.J. The finite dose technique as a valid in vitro model for the study of percutaneous absorption. Curr.Prob.Dermatol., 7,(1978), 58-68.

51. Busse,M.J., Hunt,P., Lees,K.A., Maggs,P.N.D. and McCarthy,T.M. Release of betamethasone derivatives from ointments-in vivo and in vitro studies. Br.J.Dermatol., 81, (1969), 103-112.

52. Chowhan,Z.T. and Pritchard,R. Release of corticoids from oleaginous ointment bases containing drug in suspension. J.Pharm.Sci., 64, (1975), 754-759.

53. Haleblain,J.K., Poulsen,B.J. and Burdick,K.H. Development of fluocinonide gel: correlation of in vitro assays, in vivo bioassays and clinical trials. Curr.Ther.Res., 22, (1977), 713-721.

54. Poulsen,B.J. The use of models in estimating vehicle effects on the activity of topical corticosteroid formulations. Br.J.Dermatol., 82 (Suppl. 6), (1970), 49-52.

55. Katz,M. and Poulsen,B.J. Corticoid, vehicle, and skin interaction in percutaneous absorption. J.Soc.Cosmet.Chem., 23, (1972), 565-590.

56. Rosvold,J., Nordby,K-E., Finholt,P. and Thune,P. Effect of formulation on in vitro release and in vivo absorption of corticosteroids from ointments II. Medd.Norsk Farm.Selsk., 44, (1982), 21-45.

57. Amundsen,B., Nenningsland,K.L., Finholt,P. and Thune,P. Effect of formulation on in vitro release and in vivo absorption of corticosteroids from ointments I. Medd.Norsk Farm.Selsk., 43, (1981), 1-15.

58. Dempski,R.E., Portnoff,J.B. and Wase,A.W. In vitro release and in vivo penetration studies of a topical steroid from nonaqueous vehicles. J.Pharm.Sci., 58, (1969), 579-582.

59. Behme,R.J., Kensler,T.T. and Brooke,D. A new technique for determining in vitro release rates of drugs from creams. J.Pharm.Sci., 71, (1982), 1303-1305.

60. Won Jun,H. and Bayoumi,S.M. A diffusion model for studying the drug release from semisolid dosage froms I. Methodology using agar gel as diffusion medium. Drug.Dev.Ind.Pharm., 12, (1986), 899-914.

61. Di Colo,G., Nannipieri,E., Serafini,M.F. and Vitale,D. A study of correlations between the release of drugs from petrolatum-based gels containing nonionic surfactants and some physical and physico-chemical characteristics of the gel systems. Int.J.Cosmet.Sci., 8, (1986), 105-113.

62. Higuchi,W.I. Diffusional models useful in biopharmaceutics. J.Pharm.Sci., 56, (1967), 315-324.

63. Hadgraft,J. Calculations of drug release rates from controlled release devices. The slab. Int.J.Pharm., 2, (1979), 177-194.

64. Turakka,L., Piepponen,T. and Kahela,P. Release of hydrocortisone and hydrocortisone acetate from topical vehicles in vitro. Labo-Pharma-Probl.Tech., 32, (1984), 540-544.

65. Kazmi,S., Kennon,L., Sideman,M. and Plakogiannis,F.M. Medicament release from ointment bases: I. indomethacin. In vitro and in vivo release release studies. Drug Dev.Ind.Pharm., 10, (1984), 1071-1083.

66. Billups,N.F. and Patel,N.K. Experiments in physical pharmacy.V. In vitro release of medicament from ointment bases. Am.J.Pharm.Educ., 34, (1970), 190-196.

67. Ashton,P. and Hadgraft,J. Some effects of a non-ionic surfactant on topical availability. J.Pharm.Pharmacol., 38 (Suppl.), (1986), 70P.

68. Gilbert,J.C., Hadgraft,J., Bye,A. and Brookes,L.G. Drug release from pluronic F-127 gels. Int.J.Pharm., 32, (1986), 233-228.

69. Ayres,J.W. and Laskar,P.A. Diffusion of benzocaine from ointment bases. J.Pharm.Sci., 63, (1974), 1402-1406.

70. Ezzedeen,F.W., Shihab,F.A. and Stohs,S.J. Release of sorbic acid from ointment bases. Int.J.Pharm., 28, (1986), 113-117.

71. Turakka,L. Release of hydrocortisone and its acetate ester from hydrogels. Acta Pharm.Fenn., 94, (1985), 17-21.

72. Malone,T., Haleblain,J.K., Poulsen,B.J. and Burdick,K.H. Development and evaluation of ointment and cream vehicles for a new topical steroid, fluclorolone acetonide. Br.J.Dermatol., 90, (1974), 187-195.

73. Mazzo,D.J., Fong,E.K.F. and Biffar,S.E. A comparison of test methods for determining in vitro drug release from transdermal delivery dosage forms. J.Pharm.Biomed.Anal., 4, (1986), 601-607.

74. Lovering,E.G. and Black,D.B. Diffusion layer effects on permeation of phenylbutazone through polydimethylsiloxane. J.Pharm.Sci., 63, (1974), 1399-1402.

75. Sato,S. and Wan Kim,S. Macromolecular diffusion through polymer membranes. Int.J.Pharm., 22, (1984), 229-255.

76. Bottari,F., Di Colo,G., Nannipieri,E., Saettone,M.F. and Serafini,M.F. Release of drugs from ointment bases II: In vitro release of benzocaine from suspension-type aqueous gels. J.Pharm.Sci., 66, (1977), 926-931.

77. Ackermann,C., Flynn,G.L. and Van Wyk,C.J. Percutaneous absorption of urea. Int.J.Cosmet.Sci., 7, (1985), 251-264.

78. Ackermann,C. and Flynn,G.L. Ether-water partitioning and permeability through nude mouse skin in vitro.I. Urea, thiourea, glycerol and glucose. Int.J.Pharm., 36, (1987), 61-66.

79. Ackermann,C., Flynn,G.L. and Smith,W.M. Ether-water partitioning and permeability through nude mouse skin in vitro.II. Hydrocortisone-n-alkyl esters, alkanols and hydrophilic compounds. Int.J.Pharm., 36, (1987), 67-71.

80. Scott,R.C., Leahy,D.E., Evans,J.R. and Bole,E. In vitro percutaneous model for quantitative structure-absorption studies. J.Pharm.Pharmacol., 38 (Suppl.), (1986), 66P.

81. Valia,K.H. and Chien,Y.W. Long-term skin permeation kinetics of estradiol: (I)effect of drug solubilizer-polyethylene glycol 400. Drug Dev.Ind.Pharm., 10, (1984), 951-981.

82. Bronaugh,R.L. and Stewart,R.F. Methods for in vitro percutaneous absorption studies. III. Hydrophobic compounds. J.Pharm.Sci., 73, (1984). 1255-1258.

83. Wester.R.C. and Maibach,H.I. Animal models for percutaneous absorption. In "Models in dermatology", 2, Maibach and Lowe, (Eds.). Karger, Basel, (1985), 159-169.

84. Sloan,K.B., Siver,K.G. and Koch,S.A.M. The effect of vehicle on the diffusion of salicylic acid through hairless mouse skin. J.Pharm.Sci., 75, (1986), 744-749.

85. Southwell,D., Barry,B.W. and Woodford,R. Variations in permeability of human skin within and between species. Int.J.Pharm., 18, (1984), 299-309.

86. Southwell,D. and Barry,B.W. Penetration enhancement in human skin; effect of 2-pyrrolidone, dimethylformamide and increased hydration on finite dose permeation of aspirin and caffeine. Int.J.Pharm., 22, (1984), 291-298.

87. Broberg,F., Brodin,A., Akerman,B. and Frank,S.G. In vitro and in vivo studies on lidocaine formulated in an o/w cream and in a polyethylene glycol ointment. Acta Pharm.Suec., 19, (1982), 229-240.

88. Waranis,R.P., Siver,K.G. and Sloan,K.B. The solubility parameter of vehicles as a predictor of relative vehicle effects on the diffusion of 6-mercaptopurine. Int.J.Pharm., 36, (1987), 211-222.

89. Langguth,P., Spahn,H. and Mutschler,E. Variations of in vitro nitroglycerin permeation through human epidermis. Pharm.Res., 3, (1986), 61-63.

90. Nakano,M. and Patel,N.K. Release, uptake, and permeation behaviour of salicylic acid in ointment bases. J.Pharm.Sci., 59, (1970), 985-988.

91. Michaels,A.S., Chandrasekaran,S.K. and Shaw,J.E. Drug permeation through human skin: theory and in vitro experimental measurement. AIChE.J., 21, (1975), 985-996.

92. Morimoto,Y., Sugibayashi,K., Hosoya,K. and Higuchi,W.I. Penetration enhancing effect of azone on the transport of 5-fluorouracil across the hairless rat skin. Int.J.Pharm., 32, (1986), 31-38.

93. Washitake,M., Takashima,Y., Tanaka,S., Anmo,T. and Tanaka,I. Drug permeation through egg shell membranes. Chem.Pharm.Bull., 28, (1980), 2855-2861.

94. Bettley,F.R. The influence of soap on the permeability of the epidermis. Br.J.Dermatol., 73, (1961), 448-454.

95. Bottari,F., Di Colo,G., Nannipieri,E., Saettone,M.F. and Serafini,M.F. Influence of drug concentration on in vitro release of salicylic acid from ointment bases. J.Pharm.Sci., 63, (1974), 1779-1783.

96. Patel,N.K. and Foss,N.E. Interaction of some pharmaceuticals with macromolecules I. Effect of temperature on the binding of parabens and phenols to polysorbate 80 and polyethylene glycol 4000. J.Pharm.Sci., 53, (1964). 94-97.

97. Barry.B.W. and Brace, A.R. Permeation of oestrone, oestradiol, oestriol and dexamethasone across cellulose acetate membrane. J.Pharm.Pharmacol., 29, (1977), 397-400.

98. Corrigan.O.I., Farvar,M.A. and Higuchi,W.I. Drug membrane transport enhancement using high energy drug polyvinylpyrrolidone (PVP) co-precipitates. Int.J.Pharm., 5, (1980). 229-238.

99. Tojo,K., Sun,Y., Ghannam,M.M. and Chien,Y.W. Characterization of a membrane permeation system for controlled drug delivery studies. AIChE J., 31, (1985), 741-746.

100. Dyer,A., Hayes,G.G. Wilson,J.G. and Catterall,R. Diffusion through skin and model systems. Int.J.Cosmet.Sci., 1, (1979), 91-100.

101. Garrett,E.R. and Chemburkar,P.B. Evaluation, control, and prediction of drug diffusion through polymeric membranes I. Methods and reproducibility of steady state diffusion studies. J.Pharm.Sci., 57, (1968). 944-949.

102. Lovering,E.G., Black,D.B. and Rowe,M.L. Drug permeation through membranes IV: effect of excipients and various additives on permeation of chlordiazepoxide through polydimethylsiloxane membranes. J.Pharm.Sci., 63, (1974), 1224-1227.

103. Langguth,P., Spahn,H., Mutschler,E. and Hubner,K. An approach to reduce the number of skin samples in testing the transdermal permeation of drugs. J.Pharm.Pharmacol., 38, (1986), 726-730.

104. Shenouda,L.S. and Mattocks,A.M. Diffusion of drugs across the isolated mesentery. J.Pharm.Sci., 56, (1967), 464-469.

105. Goldberg,A.H. and Higuchi,W.I. Improved method for diffusion coefficient determination employing the silver membrane filter. J.Pharm.Sci., 57, (1968), 1583-1585.

106. Wurster,D.E., Ostrenga,J.A. and Matheson,L.E. Sarin transport across excised human skin I: permeability and adsorption characteristics. J.Pharm.Sci., 68, (1979), 1406-1409.

107. Durrheim,H., Flynn,G.L., Higuchi,W.I. and Behl,C.R. Permeation of hairless mouse skin I: experimental methods and comparison with human epidermal permeation by alkanols. J.Pharm.Sci., 69, (1980). 781-786.

108. Touitou,E. and Abed,L. The permeation behaviour of several membranes with potential use in the design of transdermal devices. Pharm.Acta Helv., 60, (1985), 193-198.

109. Touitou.E. Transdermal delivery of anxiolytics: in vitro skin permeation of midazolam maleate and diazepam. Int.J.Pharm., 33, (1986). 37-43.

110. Touitou.E. and Abed.L. Effect of propylene glycol, azone and N-decylmethyl sulphoxide on skin permeation kinetics of 5-fluorouracil. Int.J.Pharm., 27, (1985) 89-98.

111. Flynn.G.L. and Smith,E.W. Memmbrane diffusionn I: design and testing of a new multifeatured diffusion cell. J.Pharm.Sci., 60, (1971). 1713-1717.

112. Galey,W.R., Lonsdale,H.K. and Nacht,S. The in vitro permeability of skin and buccal mucosa to selected drugs and tritiated water. J.Invest.Dermatol., 67, (1976), 713-717.

113. Barry,B..Harrison,S.M. and Dugard,P.H. Correlation of thermodynamic activity and vapour diffusion through human skin for the model compound, benzyl alcohol. J.Pharm.Pharmacol., 37, (1985). 84-90.

114. Harrison,S.M., Barry,B.W. and Dugard,P.H. Benzyl alcohol vapour diffusion through human skin: dependence on thermodynamic activity in the vehicle. J.Pharm.Pharmacol., 34 (Suppl.), (1982), 36P.

115. Chien,Y.W. and Valia,K.H. Development of a dynamic skin permeation system for long-term permeation studies. Drug Dev.Ind.Pharm., 10, (1984), 575-599.

116. Valia,K.H. and Chien,Y.W. Long-term skin permeation kinetics of estradiol: (II) kinetics of skin uptake, binding, and metabolism. Drug Dev.Ind.Pharm., 10, (1984), 991-1015.

117. Tojo,K., Masi,J.A. and Chien,Y.W. Hydrodynamic characteristics of an in vitro drug permeation cell. Ind.Eng.Chem.Fundam., 24, (1985), 368-373.

118. Tojo,K., Ghannam,M.M. Sun,Y. and Chien,Y.W. In vitro apparatus for controlled release studies and intrinsic rate of permeation. J.Controlled Release, 1, (1985), 197-203.

119. Tojo.K., Sun,Y., Ghannam,M.M. and Chien,Y.W. Characterization of a membrane permeation system for controlled drug delivery studies. AIChE.J., 31, (1985), 741-746.

120. Ghannam,M.M., Tojo,K. and Chien,Y.W. Kinetics and thermodynamics of drug permeation throughsilicone elastomers (I) effect of penetrant hydrophilicity. Drug Dev.Ind.Pharm., 12, (1986). 303-325.

121. Liu,J.C., Tan,E.L. and Chien,Y.W. Dissolution kinetics and rate controlling mechanisms. Drug Dev.Ind.Pharm., 12, (1986), 1357-1370.

122. Tojo.K., Sun,Y., Ghannam.M. and Chien,Y.W. Simple evaluation method of intrinsic diffusivity for membrane moderated controlled release. Drug Dev.Ind.Pharm., 11, (1985), 1363-1371.

123. Lee,C-L., Ulman,K.L. and Larson,K.R. Kinetics and thermodynamics of drug permeation through silicone elastomers IV. Effect of polymer backbone structure. Drug Dev.Ind.Pharm., 12, (1986), 369-383.

124. Lee,C-L., Ulman,K.L. and Larson,K.R. Kinetics and thermodynamics of drug permeation through silicone elastomers III. Effect of alkyl substituent (R) in (MeRSiO)x polymer. Drug Dev.Ind.Pharm., 12, (1986). 349-368.

125. Liu,J.-C., Sun.Y., Tojo,K. and Chien,Y.W. Membrane permeation kinetics of nortestosterone: effect of methyl groups on thermodynamics. Int.J.Pharm., 25, (1985), 265-274.

126. Sun,Y., Tojo,K. and Chien,Y.W. Kinetics and thermodynamics of drug permeation through silicone elastomers (II) effect of penetrant lipophilicity. Drug dev.Ind.Pharm., 12, (1986), 327-348.

127. Akhter,S.A. and Barry,B.W. Flurbiprofen penetratiion and enhancement through cadaver skin: value of "in vivo mimic" approach with low enhancer concentrations. J.Pharm.Pharmacol., 35 (Suppl.), (1983), 30P.

128. Akhter.S.A. and Barry,B.W. Penetration enhancers in human skin - effect of oleic acid and azone on flurbiprofen permeation. J.Pharm.Pharmacol., 36 (Suppl.), (1984), 7P.

129. Akhter,S.A., Barry,B.W. and Meyer,M.C. Absorption through cadaver skin of ibuprofen, applied as dry films; effects of solvents. J.Pharm.Pharmacol., 34 (Suppl.), (1982), 34P.

130. Akhter,S.A. and Barry,B.W. Absorption through human skin of ibuprofen and flubiprofen; effect of dose variation, deposited drug films, occlusion and the penetration enhancer N-methyl-2-pyrrolidone. J.Pharm.Pharmacol., 37, (1985), 27-37.

131. Tanaka,S., Takashima,Y., Murayama,H. and Tsuchiya,S. Studies on drug release from ointments. V. Release of hydrocortisone butyrate propionate from topical dosage forms to silicone rubber. Int.J.Pharm., 27, (1985), 29-38.

132. Foreman,M.I., Kelly,I. and Lukowiecki,G.A. A method for the measurement of diffusion constants suitable for studies of non-occluded skin. J.Pharm.Pharmacol., 29, (1977), 108-109.

133. Michniak,B.B. Advances in topical corticosteroid therapy. Int.J.Cosmet.Sci., 5, (1983), 277-291.

134. Michniak-Mikolajczak,B.B. and Bodor,N. A study of the penetration of five novel synthetic steroids through hairless mouse skin in vitro. Int.J.Cosmet.Sci., 7, (1985), 175-179.

135. Foreman,M.I., Clanachan,I. and Kelly,I.P. The diffusion of nandrolone through occluded and non-occluded human skin. J.Pharm.Pharmacol., 30, (1978), 152-157.

136. Scheuplein,R.J. and Ross,R.W. Mechanism of percutaneous absorption V. Percutaneous absorption of solvent deposited solids. J.Invest.Dermatol., 62, (1974), 353-360.

137. Young-Harvey,J.A., Rae,I.D. and Pitman,I.H. Oxazolidines: prodrugs for the delivery of B-aminoalcohols through human skin from aqueous solution. Int.J.Pharm., 30, (1986), 151-160.

138. Chowhan,Z.T. and Pritchard,R. Effects of surfactants on percutaneous absorption of naproxen I: comparison of rat, rabbit and human excised skin. J.Pharm.Sci., 69, (1978), 1272-1274.

139. Ostrenga,J., Steinmetz,C. and Poulsen,B. Significance of vehicle composition I: relationship between topical vehicle composition, skin penetrability, and clinical efficacy. J.Pharm.Sci., 60, (1971), 1175-1179.

140. Chow,D.S-L., KKaka,I. and Wang,T.I. concentration-dependent enhancement of 1-dodecylazacycloheptan-2-one on the percutaneous penetration kinetics of triamcinolone acetonide. J.Pharm.Sci., 73, (1984), 1794-1799.

141. Iyer,B.V. and Vasavada,R.C. In vitro skin permeation of triamcinolone acetonide from lanolin alcohol-ethyl cellulose films. Int.J.Pharm., 3, (1979), 247-260.

142. Ogiso,T., Ito,Y., Iwaki,M. and Atago,H. Absorption of indomethacin and its calcium salt through rat skin: effect of penetration enhancers and relationship between in vivo and in vitro penetration. J.Pharmacobio-Dyn., 9, (1986). 517-525.

143. Chien.Y.W., Keshary,P.R., Huang,Y.C. and Sarpotdar,P.P. Comparitive controlled skin permeation of nitroglycerin from marketed transdermal delivery systems. J.Pharm.Sci., 72, (1983), 968-970.

144. Crutcher.W. and Maibach,H.I. The effect of perfusion rate on in vitro percutaneous penetration. J.Invest.Dermatol., 53, (1969), 264-269.

145. Marzulli,F.N. Barriers to skin penetration. J.Invest.Dermatol., 39, (1962). 387-393.

146. Aguiar.A.J. and Weiner.M.A. Percutaneous absorption studies of chloramphnicol solutions. J.Pharm.Sci., 58. (1969). 210-215.

147. Ainsworth,M. Methods for measuring percutaneous absorption. J.Soc.Cosmet.Chem., 11, (1960). 69-78.

148. Hawkins,G.S. and Reifenrath,W.G. Influence of skin source, penetration cell fluid, and partition coefficient on in vitro skin penetration. J.Pharm.Sci., 75, (1986), 378-381.

149. Okamoto,H., Komatsu,H., Hashida,M. and Sezaki,H. Effects of B-cyclodextrin and di-O-methyl-B-cyclodextrin on the percutaneous absorption of butylparaben, indomethacin and sulfanilic acid. Int.J.Pharm., 30, (1986), 35-45.

150. Akhter,S.A. and Barry,B.W. Classification of penetration enhancers for human skin: effect on mannitol and octanol absorption. J.Pharm.Pharmacol., 35 (Suppl.), (1983), 28P.

151. Bronaugh,R.L. and Stewart.R.F. Methods for in vitro percutaneous absorption studies IV: the flow through diffusion cell. J.Pharm.Sci., 74, (1985), 64-67.

152. Bronaugh,R.L. and Stewart,R.F. Methods for in vitro percutaneous absorption studies VII: use of excised human skin. J.Pharm.Sci., 75, (1986), 1094-1097.

153. Bronaugh,R.L. and Stewart.R.F. Methods for in vitro percutaneous absorption studies VI: preparation of the barrier layer. J.Pharm.Sci., 75, (1986). 487-491.

154. Elias,P.M., Brown,B.E. and Ziboh.V.A. The permeability barrier in essential fatty acid defficiency: evidence for a direct role for linoleic acid in barrier function. J.Invest.Dermatol., 74, (1980), 230-233.

155. Bronaugh,R.L., Stewart,R.F. . Congdon,E.R. and Giles,A.L. Methods for in vitro percutaneous absorption studies I. Comparison with in vivo results. Toxicol.Appl.Pharmacol., 62, (1982), 474-480.

156. Cooper,E.R., Merritt,E.W. and Smith,R.L. Effect of fatty acids and alcohols on the penetration of acyclovir across human skin in vitro. J.Pharm.Sci., 74, (1985), 688-689.

157. Kligman,A.M. and Christophers,E. Preparation of isolated sheets of human stratum corneum. Arch.Dermatol., 88, (1963). 702-705.

158. Lee,G. and Parlicharla.P. An examination of excised skin tissues used for in vitro membrane permeation studies. Pharm.Res., 3, (1986), 356-359.

159. Scheuplein,R.J. and Blank,I.H. Mechanism of percutaneous absorption. IV. Penetration of non-electrolytes (alcohols) from aqueous solution and from pure liquid. J.Invest.Dermatol., 60, (1973). 286-296.

160. Scott,R.C., Walker,M. and Dugard,P.H. In vitro percutaneous absorption experiments: a technique for the production ofintact epidermal membranes from rat skin. J.Soc.Cosmet.Chem., 37, (1986), 35-41.

161. Ferguson,J. A method to facilitate the isolation and handling of stratum corneum. Br.J.Dermatol., 96, (1977), 21-23.

162. Rietschel,R.L. and Akers,W.A. Effects of harvesting techniqes on hydration dynamics: gravimetric studies of stratum corneum. J.Soc.Cosmet.Chem., 29, (1978), 777-782.

163. Kitano,Y. and Okada,N. Separation of the epidermal sheet by dipase. Br.J.Dermatol., 108, (1983). 555-560.

164. Epstein,E.H., Munderloh,N.H. and Fukuyama,K. Dithiothreitol separation of newborn rodent dermis and epidermis. J.Invest.Dermatol., 73, (1979), 207-210.

165. MacKenzie,I.C. and Linder,J.E. An examination of cellular organization within the stratum corneum by silver staining method. J.Invest.Dermatol., 61, (1973), 245-250.

166. Foreman,M.I., Clanachan,I. and Kelly,I.P. Diffusion barriers in skin-a new method of comparison. Br.J.Dermatol., 108, (1983), 549-553.

167. Middleton,J.D. The mechanism of water binding in stratum corneum. Br.J.Dermatol., 80, (1968). 437-450.

168. Foley,F.F. and Aftonomos,B. The use of pronase in tissue culture: a comparison with trypsin. J.Cell Physiol., 75, (1970), 159-161.

169. Elias,P.M., Cooper,E.R., Korc,A. and Brown,B.E. Percutaneous transport in relation to stratum corneum structure and lipid composition. J.Invest.Dermatol., 76, (1981), 297-301.

170. Idson,B. Percutaneous absorption. J.Pharm.Sci., 64, (1975). 901-924.

171. Frosch,P.J., Wendt,H. and Kligman,A.M. Evaluation of corticosteroid atrophy by the ammonium hydroxide blister model. Arch.Dermatol.Res., 261, (1978), 108.

172. Kiistala,U. and Mustakallio,K.K. Dermo-epidermal separation with suction. Electron microscopic and histochemical study of initial events of blistering on human skin. J.Invest.Dermatol., 48, (1967). 466-477.

173. Hellum,K.B., Schreiner,A., Digranes,A. and Bergman,I. Skin blisters produced by suction: a new model for studies of penetration of antibiotics in humans. Curr.Chemother., Proc.10th Int.Congr.Chemother., (1978) 620-622.

174. Polano,M.K., Ponec,M., Smeenk,G. and Hendrikse,J.C.M. In "Advances in biology of the skin", XII, Montagna,W., VanScott,E.J. and Stoughton,R.B. (Eds.). Appleton-Century-Crofts, New York, (1972). 325-328.

175. Rothbert,S. and Axilrod,G.D. The action of trypsin on insoluble epidermal keratin and the nature of the epidermal proteins solubilized. Biochem.Med., 2, (1968), 1-11.

176. Jensen,H.M. and Mottet,N.K. Ultrastructoral changes in keratinizing epithelium following trypsinization, epidermal detachment, and apposition to mesenchymes. J.Cell.Sci., 6, (1970), 511-534.

177. Anderson,R.L., Cassidy,J.M., Hansen,J.R. and Yellin,W. Hydration of stratum corneum. Biopolymers, 12, (1973), 2789-2802.

178. Bronaugh,R.L., Stewart,R.F. and Congdon,E.R. Differences in permeability of rat skin related to sex and body site. J.Soc.Cosmet.Chem., 34, (1983), 127-135.

179. Bartek,M.J. and La Budde,J.A. Percutaneous absorption in vitro. In "Animal models in dermatology", Maibach.H.I. (Ed.), Churchill Livingstone, New York, (1975), 103-120.

180. McGreesh.A.H. Percutaneous toxicity. Toxicol.Appl.Pharmacol., Suppl 2, (1965), 20-26.

181. Bronaugh,R.L. and Maibach.H.I. In vitro models for human percutaneous absorption. In "Models in dermatology", 2, Maibach,H.I. and Lowe, (Eds.). Karger, Basel, (1985), 178-188.

182. Bronaugh.R.L., Stewart,R.F. and Congdon,E.R. Methods for in vitro percutaneous absorption studies II. Animal models for human skin. Toxicol.Appl.Pharmacol., 62, (1982). 481-488.

183. Walker.M., Dugard.P.H. and Scott,R.C. In vitro percutaneous absorption studies: a comparison of human and laboratory species. Hum.Toxicol., 2, (1983). 561-562.

184. Scott.R.C., Walker.M. and Dugard,P.H. A comparison of the in vitro permeability properties of human and some laboratory animal skins. Int.J.Cosmet.Sci., 8, (1986). 189-194.

185. Gligman.A.M. A biological brief on percutaneous absorption. Drug.Dev.Ind.Pharm., 9, (1983), 521-560.

186. Baker,H. and Kligman,A.M. A simple in vivo method for studying the permeability of the human stratum corneum. J.Invest.Dermatol., 48, (1967), 273-274.

187. Holbrook,K.A. and Odland,G.F. Regional differences in the thickness (cell layers) of the human stratum corneum: an ultrastructural analysis. J.Invest.Dermatol., 62, (1974), 415-422.

188. Liang,W-Q., Petersen,R.V. and Wan Kim,S. Drug permeation through polymer-skin membranes. Acta Pharm.Technol., 31, (1985), 210-214.

189. Green,M.C. In "Genetic variants and strains of the laboratory mouse". Green,M.C. (Ed.), Gustav Fischer Verlag, New York, (1981), 117-118.

190. Behl,C.R., Flynn,G.L., Linn,E.E. and Smith,W.M. Percutaneous absorption of corticosteroids : age, site, and skin-sectioning influences on rates of permeation of hairless mouse skin by hydrocortisone. J.Pharm.Sci., 73, (1984). 1287-1290.

191. Flynn.G.L., Durrheim.H., and Higuchi,W.I. Permeation of hairless mouse skin II: membrane sectioning techniques and influence on alkanol permeabilities. J.Pharm.Sci., 70, (1981), 52-56.

192. Stoughton.R.B. Animal models for in vitro percutaneous absorption. In "Animal models in dermatology", Maibach H.I. (Ed.), Churchill Livingstone, New York, (1975), 121-132.

193. Stoughton.R.B. Bioassay system for formulations of topically applied glucocorticoids. Arch.Dermatol., 106, (1972). 825-830.

194. Yu,C.D., Gordon.N.A., Fox.J.L., Higuchi,W.I. and Ho,N.F.H. Physical model evaluation of topical prodrug delivery-simultaneous transport and bioconversion of vidarabine-5'-valerate V: Mechanistic analysis of influence of nonhomogeneous enzyme distributions in hairless mouse skin. J.Pharm.Sci.. 69, (1980). 775-780.

195. Ando,H.Y., Ho,N.F.H. and Higuchi,W.I. In vitro estimates of topical bioavailability. J.Pharm.Sci., 66, (1977). 755-757.

196. Dugard,P.H., Scott,R.C., Ramsey,J.D., Mawdsley,S.J. and Rhodes,C. Percutaneous absorption of phthalate esters: in vitro experiments on human and rat epidermal membranes. Hum.Toxicol., 2, (1983). 561-562.

197. Marzulli,F.N., and Maibach,H.I. Relevance of animal models: The hexachlorophene story. In "Animal models in dermatology", Maibach,H.I. (Ed.), Churchill Livingstone, New York, (1975), 156-167.

198. Wepierre,J., Corroller,M. and Didry,J.R. Distributlon and dissociation of benzoyl peroxide in cutaneous tissue after application on the skin in the hairless rat. Int.J.Cosmet.Sci., 8, (1986). 97-104.

199. Picton,W., Devitt,H. and Forgie,M.A. Practical applications of the Quantimet 720, image analysing computer, in the field of investigative dermatology. Br.J.Dermatol., 95, (1970), 341-345.

200. Andersen.K.E., Maibach,H.I. and Anjo,M.D. The guinea pig: an animal model for human skin absorption of hydrocortisone, testosterone and benzoic acid. Br.J.Dermatol., 102, (1980), 447-453.

201. Creasey,N.H., Battensby.J. and Fletcher,J. Factors affecting the permeability of the skin. Curr.Prob.Dermatol., 7, (1978), 95-106.

202. Campbell,P., Watanabe,T. and Chandrasekaran,S.K. Comparison of in vitro skin permeability of scopolamine in rat, rabbit, and man. Fed.Proc., 35, (1976), 639.

203. Wester,R.C. and Maibach,H.I. Relationship of topical dose and percutaneous absorption in rhesus monkey and man. J.Invest.Dermatol., 67, (1976), 518-520.

204. Wester,R.C. and Maibach,H.I. Percutaneous absorption in the rhesus monkey compared to man. Toxicol.Appl.Pharmacol., 32, (1975), 394-398.

205. Wester,R.C., Noonan,P.K. and Maibach,H.I. Effect of salicylic acid on the percutaneous absorption of hydrocortisone. In vivo studies in the rhesus monkey. Arch.Dermatol., 114, (1978), 1162-1164.

206. Wester,R.C., Noonan,P.K. and Maibach,H.I. Variations in percutaneous absorption of testosterone in the rhesus monkey due to anatomic site of application and frequency of application. Arch.Dermatol.Res., 267, (1980). 229-235.

207. Kneczke.M., Landersjo,L., Lundgren,P. and Fuhrer,C. In vitro release of salicylic acid from two different qualities of white petrolatum. Acta Pharma.Suec., 23, (1986). 193-204.

208. Turakka.L., Kontra.K. and Kyllonen,A-M. Release of hydroxy benzoic acids from a triglyceride vehicle containing surfactants. Pharm.Acta Helv., 58, (1983). 153-159.

209. Ceschel.G.C. and Tartarini,A. Studio dell'assorbimento in vitro di prodotti topici mediante una membrana di nuova produzzione-correlazione con altre metodiche in vitro e in vivo. Boll.Chim.Farm., 122, (1983), 177-185.

210. Corrigan,O.I., Farvar,M.A. and Higuchi,W.I. Drug membrane transport enhancement using high energy drug polyvinylpyrrolidone co-precipitates. Int.J.Pharm., 5, (1980), 229-238.

211. Barry,B.W. and Woodford,R. Activity and bioavailability of topical steroids. In vivo/in vitro correlations for the vasoconstrictor test. J.Clin.Pharm., 3, (1978), 43-65.

212. Ceschel,G.C. and De Filippis,P. diffusion through artificial lipid barriers of two forms of betamethasone and correlation with in vivo data. Pharm.Acta Helv., 56, (1981), 291-295.

213. Viegas,T.X., Kibbe,A.H., Hikal,A.H., Cleary,R.W. and Jones,A.B. An in vitro method of evaluating tolnaftate release from topical powder. Pharm.Res., 3, (1986), 88-92.

214. De Meere,A.L.J. and Tomlinson,E. Physicochemical description of the absorption rate of a solute between water and 2,2,4-trimethylpentane. Int.J.Pharm., 22, (1984), 177-196.

215. Olanoff,L., Koinis,T. and Anderson,J.M. Controlled release of tetracycline I: In vitro studies with a trilaminate 2-hydroxyethyl methacrylate-methyl methacrylate system. J.Pharm.Sci., 68, (1979). 1147-1150.

216. Zentner,G.M., Cardinal,J.R., Feijen,J. and Suk-Zu Song. Progestin permeation through polymer membranes IV: mechanism of steroid permeation and functional group contributions to diffusion through hydrogel films. J.Pharm.Sci., 68, (1979), 970-975.

217. Di Colo,G., Carelli.V., Giannaccini,B., Serafini.M.F. and Bottari,F. Vehicle effects in percutaneous absorption: In vitro study of influence of solvent power and microscopic viscosity of vehicle on benzocaine release from suspension hydrogels. J.Pharm.Sci., 69, (1980), 387-391.

218. Flynn,G.L. and Roseman,T.J. Membrane diffusion II. Influence of physical adsorption on molecular flux through heterogeneous dimethylpolysiloxane barriers. J.Pharm.Sci., 60, (1971), 1788-1796.

219. Garrett,E.R. and Chemburkar,P.B. Evaluation, control, and prediction of drug diffusion through polymeric membranes II. Diffusion of aminophenones through silastic membranes: a test of the pH-partition hypothesis. J.Pharm.Sci., 57, (1968). 949-959.

220. Roseman,T.J. and Higuchi,W.I. Release of medroxyprogesterone acetate from a silicone polymer. J.Pharm.Sci., 59, (1970). 353-357.

221. Haleblain,J., Runkel,R., Mueller.N., Christopherson,J. and Ng,K. Steroid release from silicone elastomer containing excess drug in suspension. J.Pharm.Sci., 60, (1971). 541-545.

222. Flynn,G.L. and Smith,R.W. Membrane diffusion III: influence of solvent composition and permeant solubility on membrane transport. J.Pharm.Sci., 61, (1972). 61-66.

223. Roseman,T.J. Release of steroids from a silicone polymer. J.Pharm.Sci., 61, (1972), 46-50.

224. Di Colo,G., Carelli,V., Nannipieri,E., Serafini,M.F. and Vitale,D. Effect of water-soluble additives on drug release from silicone rubber matrices.II. Sustained release of prednisolone from non-swelling devices. Int.J.Pharm., 30, (1986). 1-7.

225. Carelli.V., Di Colo,G. and Nannipieri,E. Effect of water-soluble additives on drug release from silicone rubber matrices. III. A study of release mechanism by differential scanning calorimetry. Int.J.Pharm., 30, (1986), 9-16.

226. Mader,W.J. and Buck,R.R. Colormetric determination of cortisone and related ketol steroids. Anal.Chem., 24, (1952). 666-667.

227. Koupparis,M.A., Walczak,K.M. and Malmstadt,H.V. Corticosteroid determination in skin preparations by a reaction rate method. J.Pharm.Sci., 68, (1979), 1479-1482.

228. Das Gupta,V. High-pressure liquid chromatographic evaluation of aqueous vehicles for preparation for prednisolone and prednisone liquid dosage forms. J.Pharm.Sci., 68, (1979), 908-910.

229. Mollica,J.A. and Strusz.R.F. Analysis of corticosteroid creams and ointments by high pressure liquid chromatography. J.Pharm.Sci., 61, (1972), 444-447.

230. Lea,A.R., Kennedy,J.M. and Low,G.K.C. Analysis of hydrocortisone acetate ointments and creams by high-performance liquid chromatography. J.Chromatogr., 198, (1980). 41-47.

231. Gordon,G. and Wood,P.R. Determination of triamcinolone acetonide in cream and suspenssion formulations by high-performance liquid chromatography. Analyst, 101, (1976). 876-882.

232. Olson,M.C. Analysis of adrenocortical steroids in pharmaceutical preparations by high-pressure liquid-liquid chromatography. J.Pharm.Sci., 62, (1973), 2001-2007.

233. Rego,A. and Nelson,B. Simultaneous determination of hydrocortisone and benzyl alcohol in pharmaceutical formulations by reversed-phase high-pressure liquid chromatography. J.Pharm.Sci., 71, (1982). 1219-1223.

234. Van Dame H.C. Quantitative determination of steroid acetates in pharmaceutical preparations by high performance liquid chromatography. J.Assoc.Off.Anal.Chem., 63, (1980), 1184-1188.

235. Leung,C.P. and Tam.S.Y.K. Determination of fluocinolone acetonide in pharmaceutical preparations by differential-pulse polarography. Analyst, 106, (1981), 100-104.

236 de Boer.H.S., Lansaat.P.H. and van Oort,W.J. Polarographic analysis for corticosteroids part 2. Determination of corticosteroids in single-component solutions, suspensions, ointments and creams. Analytica Chimica Acta, 108, (1979), 389-393.

237. Bailey.E. The gas liquid chromatographic analysis of some C-20,21-dihydroxycorticosteroids as C-20,21-acetonides. Steroids, 10, (1967), 527-545.

238. Amin.M. and Schneider.P.W. Quantitative determination of steroids in semi-solid pharmaceutical preparations by using high-performance liquid chromatography. Analyst, 103, (1978), 1076-1079.

239. Kirschbaum.J., Kerr.L., Perlman.S. and O'Laughlin,R. Quantification of steroids in topical formulations by HPLC. Anal.Chem.Symp.Ser., 23, (1985), 445-451.

240. Beyer,W.F. Advantages of a common column and common mobile phase system for steroid analyses by normal phase high-performance liquid chromatography. Pharmacopeial Forum, (July-Aug. 1982), 2232-2236.

241. Van de Vaart,F.J., Hulshoff,A. and Indemans,A.W.M. Analysis of creams; a critical review. Pharm.Weekbl , 115, (1980), 1429-1438.

242. Van De Vaart,F.J., Indemans,A.W.M., Hulshoff,A. and Lake,O.A. The application of chromatography to the analysis of pharmaceutical creams. Chromatographia, 16, (1982), 247-250.

243. Metha.A.C., Calvert,R.T. and Ryatt,K.S. Betamethasone 17-valerate. An investigation into its stability in Betnovate after dilution with emulsifying ointment: quantitation of degradation

products. Br.J.Pharm.prac., 3, (1982), 10-13.

244. Ohshiro,S., Kyan,N. and Fujimoto,K. HPLC determination of betamethasone 17-valerate in commercial ointment and admixtures. Byoin Yakugaku (BYYAD), 10 (1984), 370-374.

245. Bailey,F. and Brittain,P.N. The quantitative determination of fluocinolone acetonide and acetonide acetate in formulated products by high pressure liquid chromatography. J.Pharm.Pharmacol., 24, (1972), 425-428.

246. Burgess,C. Rapid reversed-phase high-performance liquid chromatographic analysis of steroid products. J.Chromatogr., 149, (1978), 233-240.

247. Li Wan Po,A., Irwin,W.J. and Yip,W.Y. High-performance liquid chromatographic assay of betamethasone 17-valerate and its degradation products. J.Chromatogr., 176, (1979), 399-405.

248. The United States Pharmacopeia, 20th Revision, United States Pharmacopeial Convention, Rockville, MD., (1980), Third Suppl., 80-81.

249. Yip,Y.W., and Li Wan Po,A. The stability of betamethasone 17-valerate in semi-solid bases. J.Pharm.Pharmacol., 31, (1979), 400-402.

250. Vukusic,I. Thin-layer chromatographic determination of betamethasone dipropionate in semi-solid pharmaceutical preparations. J.Chromatogr., 243, (1982), 131-138.

251. Van de Vaart,F.J., Hulshoff,A. and Indemans,A.W.M. Analysis of creams V. Application of thin layer chromatography. Part II. Pharm.Weekbl.Sci.Ed., 5, (1983), 113-118.

252. Tokunaga,H., Kimura,T. and Kawammura,J. Determination of glucocorticoids by liquid chromatography. III. Application to ointments and a cream containing cortisone acetate, dexamethasone acetate, fluorometholone, and betamethasone valerate. Chem.Pharm.Bull., 32, (1984), 4012-4016.

253. Tokunaga,H., Kimura,T. and Kawammura,J. Determination of glucocorticoids by liquid chromatography. II. Application to ointments and eye ointments containing hydrocortisone, hydrocortisone acetate, fluocinolone acetate, and prednisolone acetate. Iyakuhin Kenkyu (Iyked), 15, (1984), 87-92.

254. Williams,P.A. and Biehl,E.R. High-pressure liquid chromatographic determination of corticosteroids in topical pharmaceuticals. J.Pharm.Sci., 70, (1981), 530-534.

255. Lake,O.A., Hulshoff,A., Van De Vaart,F.J. and Indemans,A.W.M. Analysis of creams IV. Application of high performance liquid chromatography. Part 1. Pharm.Weekbl.Sci.Ed., 5, (1983) 15-21.

256. Erni,F., Keller,H.P., Morin,C. and Schmitt,M. Application of column switching in high-performance liquid chromatography to on-line sample preparation for complex separations. J.Chromatogr., 204, (1981) 65-76.

257. Benjamin E.J. and Conley,D.L. On-line HPLC method for clean-up and analysis of hydrocortisone and sulconazole nitrate in a cream. Int.J.Pharm., 13, (1983), 205-217.

258. Conley D.L. and Benjamin E.J. Automated high-performance liquid chromatographic column switching technique for the on-line clean-up and analysis of drugs in topical cream formulations. J.Chromatogr., 257, (1983), 337-344.

259. Apffel,J.A., Alfredson,T.V. and Majors,R.E. Automated on-line multi-dimensional high-performance liquid chromatographic techniques for the clean-up and analysis of water soluble samples. J.Chromatogr., 206, (1981), 43-57.

260. Little,C.J., Stahel,O., Lindner,W. and Frei,R.W. Column switching techniques in modern HPLC. Int.Laborat., 14, (1984) 26-34.

261. Majors,R.E. Multidimensional high performance liquid chromatography. J.Chromatogr., 167, (1978), 17-30.

262. Smith,E.W., Haigh,J.M. and Kanfer,I. A stability-indicating HPLC assay with on-line clean-up for betamethasone 17-valerate in topical dosage forms. Int.J.Pharm., 27, (1985), 185-192.

263. Munson,J.W. and Wilson,T.D. High-performance liquid chromatographic determination of hydrocortisone cypionate: method development and characterization of chromatographic behaviour. J.Pharm.Sci., 70, (1981) 177-182.

264. Kenley,R.A., Chaudhry,S. and Visor,G.C. An automated, column-switching HPLC method for analyzing active and excipient materials in both cream and ointment formulations. Drug Dev.Ind.Pharm , 11, (1985), 1781-1796.

265. Hammarstrand,K. Internal standards in gas chromatography. Varian Instrument Applications, 10, (1976), 10-11.

266. Upton,L.M., Townley,E.R. and Sancilio,F.D. Analysis of steroid phosphates by high pressure liquid chromatography: betamethasone sodium phosphate. J.Pharm.Sci., 67, (1978), 913-916.

267. Blank,I.H., Moloney,J., Emslie,A.G., Simon,I. and Apt,C. The diffusion of water across the stratum corneum as a function of its water content. J.Invest.Dermatol., 82, (1984), 188-194.

268. Evans,J.R. and Hughes,C. In vitro methodology for investigating quantitative relationships between skin permeation and compound structure. J.Pharm.Pharmacol., 38 (Suppl.), (1986), 68P.

269. Scheuplein,R.J. Mechanism of percutaneous absorption II. Transient diffusion and the relative importance of various routes of skin penetration. J.Invest.Dermatol., 48, (1967), 79-88.

270. Idson,B. Hydration and percutaneous absorption. Curr.Prob.Dermatol., 7, (1979), 132-141.

271. McKenzie,A.W. and Stoughton.R.B. Methods for comparing percutaneous absorption of steroids. Arch.Dermatol., 86, (1962). 608-610.

272. Meyer.E. M.Sc. Thesis: Comparative bioavailability and ranking of topical corticosteroid formulations. School of Pharmaceutical Sciences, Rhodes University, Grahamstown, South Africa. (1985). pp 38-47,55-61, 68-76.

273. Haigh.J.M. and Kanfer,I. Assessment of topical corticosteroid preparations: the human skin blanching assay. Int.J.Pharm., 19, (1984), 245-262.

274. Meyer.E., Kanfer.I. and Haigh,J.M. Comparative blanching activities of some topical corticosteroid containing lotions. S.Afr.Pharm.J., 48, (1981), 551-552.

275. Poulsen,B.J., Burdick,K. and Blesser,S. Paired comparison vasoconstrictor assays. Arch.Dermatol., 109, (1974) 367-371.

276. Wendt,H. and Frosch,P.J. In "Clinico-pharmacological models for the assay of corticoids", Karger, Basel, (1982). 13-60.

277. Bundgaard.H. and Hansen.J. Studies on the stability of corticosteroids VI. Kinetics of the rearrangement of betamethasone 17-valerate to the 21-valerate ester in aqueous solution. Int.J.Pharm., 7, (1981) 197-203.

288. British Pharmaceutical Codex, 11th edition, Pharmaceutical Press, London, (1979), 155.

289. Dunn,O.J. In "Basic statistics: a primer for the biomedical sciences" 2nd Ed., John Wiley & Sons, New York, (1977), 81-102.

290. Coleman,G.L. M.Sc. Thesis: The in vivo and quantitative assessment of topical corticosteroid formulations. School of Pharmaceutical Sciences, Rhodes University, Grahamstown, South Africa. (1977). 120-129.

291. Magnus.A.D., Haigh,J.M. and Kanfer,I. Assessment of some variables affecting the blanching activity of betamethasone 17-valerate cream. Dermatologica, 160, (1980), 321-327.