A SPECTROPHOTOFUOROMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF HYDROCORTISONE ABSORPTION IN THE SKIN FROM DERMATOLOGICAL MEDICATIONS

by

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We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

July, 1967
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ABSTRACT

The assessment of drug activity in skin is hampered by the lack of adequate methods for quantitative measurement of drug penetration in skin. Therefore, a new method was developed with the following features. The drug, hydrocortisone, chosen for its clinical and pharmaceutical importance, was applied under occlusive dressing for varying time intervals. Small skin samples, which ranged from 0.5 to 20 mg in size, were removed, in vivo, from humans or animals by a new dermatome serial slicing technique. The amount of hydrocortisone absorbed into the epidermis and/or dermis was determined by a new spectrophotofluorometric procedure, based on an older technique described for blood. The tissue levels were then correlated with simultaneously determined skin function measurements.

The assay was done at 470 μm and 525 μm excitation and emission wavelengths, respectively. Hydrocortisone was extracted from homogenized tissue with dichloromethane. Fluorescence was developed with 85:15 w/w sulphuric acid - ethyl alcohol solution.

Hydrocortisone was found in epidermis, but not in dermis. These results confirm current theories on hydrocortisone depot formation in the epidermis. Among the 14 patients examined, the concentration of hydrocortisone in epidermis varied greatly from 20 μg to 358 μg per Gm wet weight of tissue. Exploratory correlations between skin respiration rates, vaso-constriction, and the amount of hydrocortisone in epidermis have been done. The analytical reliability has been discussed.

Signature of Supervisor..............................
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INTRODUCTION

This investigation surveys methods for quantitative measurement of drugs in skin and proposes a spectrophotofluorometric method, here developed for hydrocortisone, for the measurement of drug penetration into the epidermis and the dermis of human and hairless mouse skin. Possibilities have been explored to correlate the concentration of hydrocortisone found in the epidermis to the effects of the drug on certain skin functions such as oxygen consumption rates and skin blanching.

The investigation was undertaken because of the following considerations. The assessment of the action of drugs in cutaneous disease as well as the assessment of the relative value of various dosage forms in treatment becomes meaningful only when the drug can be measured in the skin in relation to the pharmacological response it evokes there. Clinical observations alone are of limited value, because, lacking a quantitative basis, they cannot be reliably compared to the results of other workers and reveal little of the underlying causes. It is the concentration of the drug present at the site of its action within the skin which determines the intensity of the pharmacological response (1). It is this concentration, in conjunction with pharmacological measurements and clinical observations, which should form the logical basis on which the physician is advised to choose the most suitable medication and the pharmacist the most suitable formulation ingredients.

It was noted that the following information gaps exist. There is little understanding of how drugs penetrate, accumulate in and affect the skin (2). A reason for this is that the methodology for both the quantitative measurement of drugs in skin and the quantitative measurement of the changes which drugs induce in skin functions is, for most purposes, inade-
quate or not available (3). The numerous studies that have been done on
the percutaneous absorption of drugs from topical dosage forms into the
bloodstream without determining what happens to the drug within the skin
itself, are often irrelevant. Topical therapy, as the name implies,
enables the drug to exert its action directly within the skin rather
than indirectly through the medium of the blood. It is noted that sys­
temic administration of drugs for their effect on skin diseases has the
disadvantage of greater incidence of systemic side effects.

The kinetics of the fate of drugs within the skin is an unex­
plored field (4). There is little agreement as to the effect of
vehicles on drug penetration. Opinions range from negligible (4) to
considerable (2, 5, 6). The choice among drugs and dosage forms re­
commended for treatment is bewildering and dosage schedules currently
used are largely empirical. This situation is particularly true for
steroid therapy. Some corticosteroids are reported to have 40 to 50
times higher anti-inflammatory potency than hydrocortisone. The inter­
pretation of the increased potency data, however, is uncertain because
the determinations have been done on the basis of an all - or - none
response of one skin function to an externally applied minimum dosage.
The potency ratios, therefore, are not comparisons, as they ought to be,
between total amounts of drugs present in skin from the moment of their
absorption to the moment of their elimination from the skin, but rather
comparisons of single point determinations at arbitrary time intervals.

It was decided, therefore,

(a) to survey the investigational methods currently in use for the
determination of drug penetration into the skin,
(b) to design on the basis of this information an improved method which would be suitable for detailed kinetic studies of drug accumulation and disappearance from localized areas of the skin and have promise of general applicability to various studies, including evaluations of commercial preparations,

(c) to demonstrate the experimental validity of the method, using a widely prescribed drug of equal clinical and theoretical importance, hydrocortisone, and

(d) to explore, on a preliminary basis, correlations between the concentration of hydrocortisone in the epidermis and simultaneously observed changes in skin functions.
In order to understand the action of a drug, it is necessary to know
(a) whether the drug can penetrate the normal skin,
(b) how far into the skin it can penetrate,
(c) with which tissues it comes in contact and
(d) the rate of penetration. (7)

Factors Determining Drug Penetration into Skin

The kinetics of drug penetration is a composite of releasing factors
in the applied phase and accumulating factors in the skin receptors (8).
Many factors occur in the applied phase alone. The chemical form of the
drug is important. Goldman (1), for instance, demonstrated that the
least soluble hydrocortisone - 21 - acetate crystals persisted in situ for
several months following intradermal injection, while the more water sol­
uble hydrocortisone alcohol crystals remained for a shorter time. Ester
salts were absorbed so rapidly as to be undetectable at biopsy. Degree
of ionization must be considered, since completely ionized substances do
not penetrate skin. Chelators may be used to aid the absorption of ions
(9). The activity coefficient of the drug is important (8). The
physicochemical properties of the corticoids affect penetrability and re­
tention of drugs in skin. For example, more polar and less mobile steroid
compounds have lower topical potency (10). Particle size reduction aids
in absorption by increasing the surface of the medication in contact with
the affected area. Micronized material is superior to coarse particles
(10). Rate of release from suspension type vehicles has not in the past
been emphasized (1). Quantification has proven difficult in such areas
of interest as the effect of ointment bases on absorption (11). That
vehicles may have a profound effect on percutaneous absorption has been
suggested by Stoughton (6) who demonstrated a 15 to 100 fold increase in corticosteroid concentration in the epidermis when drugs were applied in a dimethysulfoxide base as compared to those applied in a control base. Use of solvents such as tetrahydrofurfuryl alcohol or dimethylacetamide produced striking improvements in the activity of hydrocortisone so that it rivaled fluorinated steroids (10). Solvents may affect follicular openings and almost certainly change activity coefficients and diffusion constants of drugs (8). The rate of absorption of acidic and basic drugs is strongly influenced by pH (8).

Some factors pertain to both the applied and the absorbing phases. The concentration gradient of the drug is an important parameter which has received little emphasis (1). Duration of drug contact and timing of applications to coincide with the receptiveness of the skin must play a significant role in the success of therapy. Occlusive dressings are known to enhance penetration. This may be a composite of drug hydration, for hydration must affect absorption, (12) and maceration effects on the skin.

Problems peculiar to the skin itself are also numerous. The epidermis is a very complex system in contrast to some other physiological membranes (4). The diffusion process through a thin membrane can be described by a single permeability index because the diffusion is constant. However, if the "membrane" is thick, as for skin, the diffusing substance accumulates within it before penetration reaches a steady state and the diffusion process becomes complicated (3). Intact skin acts as a semipermeable membrane with negative electrical charge which prevents penetration of anions and possibly resists cations electrostatically. The semipermeable area has been localized in the stratum conjunctum. Opinions vary as to whether this is a single barrier rate controlling step or a complex of many layers of differing diffusion capacities (2). Feldman and
Maibach (13) suggest that there is a significant barrier remaining after removal of the stratum corneum. Appendages such as pilosebaceous follicles can theoretically permit circumvention of the barrier because they open to the surface and end below the barrier. Reiss (9) states tacitly that the preferred route is through follicles. Other have felt that the role of the appendages may have been overemphasized (14). Normal skin exists in a large number of permeability states. Absorption in normal skin also depends upon anatomical site. Corticosteroids are more effective in areas of thinner skin such as eyelids, face or neck rather than on the thicker horny layer of palms and soles (10).

Diseased or injured skin presents further problems. Denuded, stripped, inflamed or roentgen irradiated skin shows more rapid and intense absorption than normal skin (9). Inflammation is known to increase percutaneous absorption of hydrocortisone, yet the amount of hydrocortisone retained in diseased skin versus the amount in normal skin has never been compared. Previous contact with lipid solvents such as chloroform or with saponins renders skin more permeable to water soluble substances while gradually diminishing the penetration of lipoidal substances (9).

Finally, it must be emphasized that, irrespective of its magnitude, the barrier function is as efficient for outward passage as for inward penetration (4). Accumulation of a drug in skin, then, depends upon the rate of release from the dosage form, the rate of uptake by the various skin layers, and the ability of the barrier to retain drugs which have entered it.
An Evaluation of Investigational Methods

Numerous methods have been recommended for the study of cutaneous absorption. Both in vitro and in vivo techniques for the measurement of drugs in skin are in current use. Diffusional models provide the quantitative relationships from which in vitro and in vivo experiments are designed (16). Histochemical techniques have proven useful in determining routes of absorption, but are limited by the number of drugs influencing enzyme systems (2, 9). Fluorescence microscopy, similarly, has a valid (9), though limited (2), role in determining absorption. Diffusion chambers find wide application. In vitro techniques have the advantage that environmental conditions are readily controlled. They have the obvious disadvantage that the tissue is dead (2).

In vivo methods frequently employ blood or urine levels for detection of absorption (11, 13, 15). Malkinson and Kirschenbaum (15) measured the rate of percutaneous absorption by noting the loss of radioactivity from applied labelled corticosteroids and the gain of radioactivity in blood and urine. After one or two hours, there was a temporary slowing of percutaneous absorption which suggested to these workers that there was an increase in tissue levels of the drug, since vasoconstriction was noted simultaneously. The evidence is clearly indirect. How very much more meaningful would the data have been had the drug been measured in the tissue of interest as well as in the ocean of fluid adjacent to it. One of the difficulties encountered in attempting by blood analysis to estimate quantitatively the amount of material which has penetrated the skin is the fact that the rate at which the material leaves the bloodstream and is excreted or stored in the tissues is seldom known (7). The method is
further handicapped because frequently the materials under study are normal body constituents prevalent in the blood (9). This may be somewhat compensated for by the use of isotopic tracers. Scott and Kalz (11) came somewhat closer to providing meaningful data with their radioautographic studies of the mode of absorption of C^{14} hydrocortisone. They were able to correlate inhibition of inflammation with maximal tissue levels of the drug. Interpretation of radioautographs is sometimes difficult. Beta emission may darken photographic film at some distance from the point of origin. (2).

Quantitative biological methods presently used in studies of skin pharmacology have limitations. In some diseases the epidermis thickens. Epidermal thickness has been used as a criterion of the drug activity of Vitamin A, but because the basal layer undulates, rather a large number of measurements are required for statistical validity. Mitotic activity has been measured (17). It is probably valid for the determination of the pharmacological effect of only very active substances (17). Vasocnstriction following application of corticosteroids has provided data on skin penetration (18, 19). Reappearance of vasoconstriction in previously treated skin upon application of occlusive dressings is taken as proof of the existence of a reservoir for the drug in the stratum corneum (19), yet chemical analysis of the stratum corneum to verify this has not been carried out. Curiously enough, there is no evidence published to support the validity of the theory that vasoconstriction effect parallels anti-inflammatory activity. Correlation of blanching intensity with the strength of the drug is only relative and skin pigmentation may interfere (2). Hair growth may be used as a pharmacological criterion. However, hair growth in animals differs from that in humans. Growth and resting patterns of the hair follicles must first be ascertained for individual animal studies. Special cell counts such as determination of melanocytes
may be of value. However, animals have no melanocytes between the hair follicles, thus limiting drug testing to ears and tails. Size and number of sebaceous glands have been used for hormone assays. Changes in skin function have been assessed by using fluorescence microscopy to follow changes in keratin, a rather successful method as an index of the value of different substances used in treatment of psoriasis (17).

The reliability of the elicitation of pharmacological actions is limited because of the lack of knowledge of the amount of material that passes through the skin, the rate of penetration, and the speed with which the material is metabolized (9). There is as yet no technique available which measures a physiological response and simultaneously correlates it with a known concentration of the drug. The mechanism of any response to local drug action will remain uncertain until the kinetics of penetration through skin are elucidated.

In the past, radioisotopes have provided the most useful assessment of drug concentration. However, radioactive techniques have the disadvantage of material supply limitation, possible hazard if measurable emission is to be established within the skin (3), unreliability as to topical changes when the drug is applied under occlusive conditions (15), and inapplicability in deeper skin regions where weak beta emission may simply be undetectable (19).
Spectrophotofluorometry in Penetration Studies

The only technique comparable in sensitivity to isotopic methods is fluorometric analysis (5). Fluorometry has three big advantages as an analytical tool. It is basically simple and measurements can be made rapidly. It is up to 1000 times more sensitive than other photometric techniques such as colourimetry or spectrophotometry. It is highly specific since every material that fluoresces has its own characteristic emission spectrum (20). In the area of pharmaceutics, it has the added advantage of being potentially applicable to commercial dosage forms of any drug which fluoresces in a measurable manner. Drugs have been qualitatively measured in skin using fluorescence techniques (21). Quantitative techniques are few in number and generally applicable methods for measuring drug concentration and pharmacological response together are virtually nonexistent.

Corticosteroids may be made to fluoresce quantitatively in strongly acidic or strongly basic media. Similar sensitivity is obtained with either reagent (22). The majority of methods involve the use of acid induced fluorescence of the hydrocortisone in blood, urine, or adrenal glands (23). Techniques vary in complexity. A two step procedure, namely, single solvent extraction followed by direct extraction into the fluorescence reagent has proven satisfactory to many workers (24, 25, 26). Others have added to this fundamental procedure sodium hydroxide prewashes to remove lipid material (27, 28, 29). An additional step or steps involving purification by solvent extraction (30, 31) renders the fluorescence later developed more specific. Others have found no significant differences between procedures which use petroleum ether prewashes and those which do not (28, 29). Any assay is divided into
(a) methods which arrive at rigorous isolation,

(b) those whose primary purpose is a simple practical clinical routine method (32). It is on the basis of purpose that the relative exactness of the assay must be decided.

The mechanism of the reaction of corticosteroids with acid has not been elucidated (33). High sensitivity seems to require a $\Delta_4^2 - 3$ - Ketone, oxygen function at C - 11, or a phenolic ring A (34). A C - 18 aldehyde appears to prevent formation of or to facilitate destruction of the fluorescence molecule. Fluorescence is exhibited by many steroids. Since different acid concentrations and different excitation wavelengths are required for optimum individual fluorescence, it is possible that selective suppression of one or more fluorescent species may be achieved, thereby avoiding the risk of mutual quenching or sensitization. The need for previous solvent separation may also be precluded (35). Adrenocortical steroids other than hydrocortisone and corticosterone may be measureable by varying conditions (36).

The extreme sensitivity of the reaction (as low as 0.005 $\mu$g of hydrocortisone) is somewhat reduced by potential interference (22). Only three natural corticosteroids give intense acid fluorescence under the conditions of the assay later discussed. These are corticosterone, hydrocortisone, and Reichstein's Compound E (22). Cholesterol presents potential interference (23). Fluorescence may be augmented, inhibited or changed by many ions, organic substances etc. (22, 34, 37). Corrective calculations have been proposed for blood analysis (32, 38, 39). Internal standards are frequently used to assess relative importance of impurities (28). There is an abundance and diversity of steroids in the skin and surface lipids. Cholesterol is a major constituent, the relative
amounts of which found in epidermis and dermis appear to vary (40).
The contribution of this and other steroids to fluorescence interference
has not been fully evaluated.

Conditions for the measurement of hydrocortisone have been well es-
tablished. Hydrocortisone is measured at excitation wavelengths close to
470 - 475 μm and emission wavelengths of 520 - 530 μm (41). Fluor-
escence spectra are meaningful for measurement only if the reference
fluorescence spectrum has been plotted by means of the same type of
apparatus and under the same conditions of measurement as the compound
to be analysed, (42). The instrument determines the exact wavelength.
The absorption spectrum of any steroid induced to fluoresce in sulphuric
acid is reported to changed with the passage of time (35). Intensity
of fluorescence is also time dependent (43). Hydrocortisone develops
fluorescence rapidly as contrasted to other steroids (35). There is no
question but that the measurement of fluorescence after a short and care-
fully timed interval substantially improves specificity, and despite the
lack of development of full fluorescence, provides more than adequate
sensitivity (23). It is obviously undesirable for fluorescence to be
rising rapidly at the time of measurement (23), which is the case at 5
minutes when nonspecific fluorogens interfere least with blood analysis
(37). After 10 to 12 minutes at 25°C., the reaction slows sufficiently
to allow a leeway of up to 1/2 minute (23). Absorption spectra and
fluorescence intensity are markedly influenced also by temperature, a
generally unevaluated parameter (23), and dilution (35,43). The dil-
uent used is generally ethyl alcohol. 60% to 75% v/v acid has variously
been used by different workers. The 70% gives rapid and intense fluo-
rescence development (33).
Preparation of any reagent for fluorometry requires attention to the quality of the constituent chemicals (44) and to temperature which increases blank values (33). Alcohol is purified to remove aldehydes and ketones (45) which contribute to fluorescence (42). No difficulties have been reported with any brand of sulphuric acid when mixed with ethyl alcohol. However, blanks may become a problem when low concentrations or small samples are used (23). The use of purified extracting solvent is mandatory (24). Chloroform and dichloromethane both give satisfactory results as solvents. The former, however, tends to form emulsions which may be difficult to separate.

The fluorometric method is readily and quickly performed, requires a minimum of technical manipulation, is capable of an acceptable degree of precision and accuracy, and its relatively great sensitivity makes it uniquely applicable where sample size is limited.
STATEMENT OF PROBLEM

Diverse methods for the study of drugs in skin exist. All, however, have one or more of several shortcomings which make the interpretation of results obtained by them uncertain. The most serious of these shortcomings is that the methods are not well suited for the study of skin pharmacodynamics and skin pharmacology because they do not directly relate the drug concentration in the skin to a measurable, pharmacological response. Without such concentration-response relationships, comparisons between relative activities of several drugs become doubtful, and the evaluation of the influence of a single drug on skin functions becomes impossible. The pharmacological responses that have been chosen by the existing methods are frequently subjective or involve all-or-none responses. Quantitative methods are few. Some are in vitro. Others, notably the radioactive isotope methods, at their present stage of development, have the drawback that they utilize measurement of either the decrease of the drug in the dosage form or else the increase of blood or urine drug levels. Disappearance of drugs from the skin surface tells nothing of where or in what quantity the drugs accumulate in the skin. Measurements of blood or urine levels are subject to the influence of complex additional variables such as excretion, distribution to other body fluids and variations in tissue storage and blood-protein binding which may invalidate the application of the data to the elucidation of the activity of the drug in skin. No method has been established which can determine with any degree of completeness the kinetics of drug penetration through skin.

Therefore it was decided to carry out the following.

(a) To select a representative drug from a group of drugs of theoretical and clinical importance for which no generally applicable method for assaying in the skin exists. Hydrocortisone alcohol was selected from the group of
anti-inflammatory corticosteroids.

(b) To develop, using this drug as a prototype, an assay technique of potential applicability to other drugs. A spectrophotofluorometric technique was chosen because of its high specificity, high sensitivity and its potential applicability to the analysis of drug release from pharmaceutical preparations including commercial preparations.

(c) To develop an in vivo skin sampling technique for the removal of intact, actively metabolizing skin samples at successive predetermined depths of the skin of humans or animals.

(d) To provide initial evidence that the method is suitable for the study of skin pharmacodynamics and skin pharmacology by measuring drug concentration under controlled conditions. Time of application, dosage form, experimental animal, depth of sample, methodology of skin washing, stripping and the presence of other drugs were varied. Measurements were made in skin slices which were, or could be, related concurrently to a pharmacological response such as respiration rate or vasoconstriction.
EXPERIMENTAL

Materials

Pharmaceuticals

The pharmaceuticals were U. S. P. grade and were used as received from the manufacturer without further purification.

Hydrocortisone, U. S. P. XVII (46) 11, 17, 21-Trihydroxyprogren-4-ene-3, 20-dione

standard solutions and ointments were prepared from Cortil\(^1\) brand of hydrocortisone alcohol. The micronized form was used.

Hydrocortisone Sodium Succinate, U. S. P. XVII (47)

Its solubility is 500 mg/ml, compared to 0.28 mg/ml for hydrocortisone alcohol (49). Buffered solutions 50mg/ml were prepared from Solu-Cortef\(^2\)

1 Trade name of Pfizer Company Ltd., Montreal, Quebec, who graciously furnished the material for this investigation.

2 Trade name of the Upjohn Company of Canada, Toronto, Ontario, who graciously supplied the sterile vials for this investigation.
brand of hydrocortisone sodium succinate and sterile water for injection.

Sterile Water for Injection, U. S. P. XVII

Hydrophilic Ointment, U. S. P. XVII (48) was prepared in this laboratory. Precautions were taken to prevent water loss during the preparation of the ointment, because loss of even small amounts of water affects the consistency of the ointment appreciably. Alteration of the rate of drug release may result. The composition of the ointment is as follows.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
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<tbody>
<tr>
<td>Methylparaben</td>
<td>0.25 gm</td>
</tr>
<tr>
<td>Propylparaben</td>
<td>0.15 gm</td>
</tr>
<tr>
<td>Sodium Lauryl Sulfate</td>
<td>10 gm</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>120 gm</td>
</tr>
<tr>
<td>Stearyl alcohol</td>
<td>250 gm</td>
</tr>
<tr>
<td>White petrolatum</td>
<td>250 gm</td>
</tr>
<tr>
<td>Purified water</td>
<td>370 gm</td>
</tr>
</tbody>
</table>

To Make about 1000 gm

The finished ointment was passed through the Pascall \textsuperscript{1} ointment mill to assure batch uniformity in texture and consistency.

2.5\% Hydrocortisone in Hydrophilic Ointment was prepared in this laboratory by levigating the powder with a small portion of base on an ointment slab and gradually incorporating the remainder of the base. The ointment was then passed through the Pascall \textsuperscript{1} ointment mill to assure dosage uniformity.

Acetulan - Solulan C - 24 Base \textsuperscript{2} was prepared in this laboratory by heating the fat derivatives up to 65\(^\circ\) C., cooling to 60\(^\circ\), then adding water at 62\(^\circ\). The mixture was stirred until it cooled to a jel. The composition of the ointment was as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetulan</td>
<td>10 ml</td>
</tr>
<tr>
<td>Solulan C 24</td>
<td>10 gm</td>
</tr>
<tr>
<td>Water</td>
<td>10 ml</td>
</tr>
</tbody>
</table>


2 American Cholesterol Products Inc., Edison, New Jersey. Personal Communication to Dr. J. O. Runikis.
2.5% Hydrocortisone in Acetulan - Solulan Base was prepared by incorporating the drug in the fused base and stirring until cool.

2% Lidocaine Hydrochloride (Xylocaine\textsuperscript{1}) with and without epinephrine 1:100,000 was used for local anaesthesia by intradermal injection. The preparation did not contribute any measurable fluorescence.

Freez - o - derm\textsuperscript{2} Aerosol

---

1 Trade name of Astra Pharmaceuticals, Cooksville, Ontario.

2 Trade Name of Ingram and Bell Limited, Vancouver, British Columbia.
Betamethasone - 17-Valerate

Dichlorisone Acetate

Fluocinolone Acetonide

Triamcinolone Acetonide

Neomycin Sulphate

1 Schering Corporation Limited, Pointe Claire, Quebec.
2 Syntex Limited, Montreal, Quebec.
3 E. R. Squibb and Sons, Ltd., Montreal, Quebec.
4 Eli Lilly and Company Limited, Indianapolis, Indiana.
Solvents and Reagents

Solvents and reagents were selected for minimum fluorescent impurities. They were found to be generally of acceptable quality. Routine purification was necessary only for the ethyl alcohol.

*Ethyl alcohol*, 100%, was purified by redistillation over 2, 4-dinitrophenylhydrazine (37) to remove aldehydes and ketones. The ethyl alcohol containing 5 grams of 2, 4-dinitrophenylhydrazine and 10 ml of hydrochloric acid per 1000 ml was refluxed for four to eight hours in a vacuum jacketed all-glass unlubricated packed distillation column with CORAD head (50). It was then distilled, the first and last 20% being discarded, then redistilled, again discarding the first and last 20%. The alcohol was stored in 100 ml well filled aluminum foil capped bottles at 2°C to minimize regeneration of aldehydes or acetone.

*Sulphuric Acid* (Allied Chemical of Canada, Ltd., Montreal, Quebec) was found to vary in quality depending on the lot. Screening was done to eliminate unsatisfactory lots.

*Fluorescence reagent*, consisting of 85 parts of sulphuric acid and 15 parts of ethyl alcohol by weight was prepared as follows. 30 grams of cold distilled ethyl alcohol was weighed into a tared glass stoppered flask packed in ice. About 90 ml of cold sulphuric acid was added slowly with constant agitation so that the solution remained cool. The solution was made up to 200 grams with cold acid. This reagent was prepared freshly just before use. Its fluorescence characteristics were reproducible from day to day so long as the same batch of acid was used. Preparation by weight was adopted because it was found that the method used by Mattingly (25),
calling for slow addition of 7 volumes of concentrated acide to 3 volumes of ethyl alcohol while cooling under the cold water tap, gave less reproducible results. This was attributed to the inherent lack of accuracy in the volumetric measurement of a viscous liquid such as sulphuric acid, and to insufficient cooling. This reagent for practical purposes is 70% acid by volume.

Dichloromethane Spectoquality (Matheson, Coleman and Bell, Norwood, Ohio) was used as received from the manufacturer. This solvent is reported by the manufacturer to have a fluorescence of 0.3 parts per billion as quinine base.

Distilled Water was obtained from a stainless steel still.

Hydrocortisone Standard Solutions were prepared by dissolving 100 mg of hydrocortisone powder in 100 ml of ethyl alcohol and making the volume up to 1000 ml with distilled water. A one to ten dilution with 10% ethyl alcohol then gave a stock solution of 10 μg/ml. The stock solution was prepared every four months, although it can probably be stored for longer (25). Dilutions were prepared daily as required.

Quinine Sulphate1 B. P. 1963 (51)

Quinine Sulphate Standard Solutions of 0.1 to 1.0 μg/ml in 0.1 N Sulphuric acid were used for instrument calibration.

1 British Drug Houses (Canada) Ltd., Toronto, Canada.
Equipment

Dermatome

The Castroviejo Electro - Keratotome\(^1\) (52) was used in modified form to meet the special requirements of this investigation. With this instrument, at histologically verified depths of 100 to 300 microns, thin skin slices were removed from the epidermis and dermis of volunteer patients. The area of the skin slices taken was varied from a fraction of a square centimeter to several square centimeters. When serial cuts through the epidermis and the dermis were done, the second cut was made with a blade narrower by a few mm to assure absence of epidermal contamination. No anaesthesia was required for single cuts. Xylocaine anaesthesia was used for serial cuts. There was little or no residual scar formation and little difficulty was encountered in obtaining several skin samples from the same patient. The modifications have been described by Stewart and Runikis (53).

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1 The Storz Instrument Company, St. Louis, Missouri.
Spectrophotofluorometer

Aminco - Bowman Spectrophotofluorometer Model 4-802\textsuperscript{1} was used. This instrument consists of a solid state Xenon lamp D. C. power supply unit, an optical unit using two grating type monochromators and a photomultiplier unit permitting the adjustment of amplification of fluorescence emission signals over a range of 1 to 1000. The output signals vary linearly during scans from 200 to 800 millimicrons. Xenon lamp and R. C. A. 1P21 photomultiplier tube of a wavelength range of 300 to 700 millimicrons were used throughout. Slit arrangement #3 (54°) was used. The cells were fused quartz of 1.3 cm path length.

The instrument is stable. Spectral peaks can be reproduced within $\pm$ 1 millimicron. Fluorescence intensity can be reproduced within $\pm$ 5\% or better. A gradual drift toward lower fluorescence intensity did occur during prolonged operation of the instrument. Occasional unexplained increases in fluorescence intensity were also observed. Periodic recalibration and frequent redetermination of standard curves were therefore necessary.

The spectrophotofluorometer measures fluorescence emission in Relative Fluorescence Intensity Values (freely abbreviated R.F.I.). This value is the product of galvanometer scale deflection and the metermultiplier setting of the photomultiplier unit of the instrument. The latter permits extension of the 0 to 100 galvanometer scale over a 1000 fold range. 0.1 $\mu$g/ml to 20 $\mu$g/ml hydrocortisone solutions were measured.

\begin{footnote}{1 American Instrument Company, Inc., Silver Spring, Maryland.}\end{footnote}
The spectrophotofluorometer has a sensitivity adjustment which permits amplification of the signal as the Xenon lamp intensity diminishes, thus permitting Relative Fluorescence Intensity values to be equated to a single standard curve for a considerable period of time. It was found, however, that a ratio calculation from a few points on a new standard curve provided more readily compared readings.

The necessity for new lamps or phototubes, and the accuracy of excitation and emission wavelength settings were ascertained with the aid of known values for standard quinine solutions as described in the manufacturer's instrument manual (54).
Analytical Accessories

Sample uniformity and purity are particularly important in fluorescence analysis because of the wide prevalence of fluorogenic impurities and because of the susceptibility of the analysis to the presence of light scattering particles. The analytical accessories used are, therefore, described in some detail. All-glass equipment was used.

Tissue homogenizers. Ground glass microhomogenizers of 0.4 to 0.5 ml total capacity consisting of a conical tube and rotating shaft were prepared as described by Santoianni (55). Homogenates were sufficiently uniform that duplicate samples gave duplicate analytical results.

Vortex Jr. Mixer.

Burettes had teflon stopcocks.

Pipettes were fitted with rubber Propipettes. Since rubber is highly fluorogenic, all pipettes were end-plugged with cotton (37).

Test tubes. Extraction tubes 15 cm x 2 cm o.d. were fitted with aluminum foil covered caps. The same size tubes fitted with glass stoppers were used for vortexing.

Whatman #1 filter paper was fluted and fitted into 60 to 120 ml glass funnels.

1 Scientific Industries Inc., Springfield, Massachusetts.
**Experimental Animals**

Hairless mice (HRS/J Strain) raised in this laboratory were used for preliminary experimental verification of the measurements before application to human subjects.
Procedures

Cleaning of Equipment

Glassware. The precaution of using Chromic Acid cleaning solution mentioned by Braunsberg and James (28) was observed. Particular attention was devoted to assure freedom from contamination by adsorbed pieces of tissue from previous analyses and by the trichromate ion. The glassware used in fluorometric analysis was kept separate from other glassware and stored under dust-free conditions.

(a) The glassware was soaked in hot ChemisolR detergent solution for 12 to 24 hours, then rinsed thoroughly with tap water and allowed to drain. Test tubes were brushed twice, once before and once after the detergent wash.

(b) Glassware was then immersed in chromic acid cleaning solution for at least 8 hours. This was followed by rinsing at least 10 times with flowing hot tap water, soaking for 1 hour in distilled water, then rinsing 4 times with distilled water. Oven drying rendered the glassware ready for use.

Quartz cells were stored in DreneR Shampoo solution as recommended by the manufacturer of the spectrophotofluorometer. They were rinsed 30 times with tap water and 30 times with distilled water, then with fluorescence reagent prior to use. Boiling nitric acid was employed when more thorough cleaning was required.

Dermatome was cleaned with 70% alcohol and autoclaved before use.
Drug Administration and Skin Dissection

The skin used for the studies was taken from the midforearm and occasionally from the back of volunteer male patients, and from the side of male hairless mice. Bilaterally symmetrical areas were used for drug treatment and for controls. The drugs were administered topically. Hydrocortisone alcohol was applied in a 2.5% concentration from Hydrophilic Ointment, U.S.P. XVII, hydrocortisone sodium succinate in a 5% concentration from a buffered solution. Controls were either the untreated skin or skin treated with the vehicle. A special control procedure was devised to check on the completeness of excess drug removal prior to sampling (see paragraph (d) below). Both control and treated areas were handled identically. Occlusive dressings were used throughout. The manner of administration and the sampling procedures were as follows.

(a) The ointment was applied by rubbing lightly with fingers or with the aid of a rubber spatula. An excess film of ointment was left on the skin surface. The solution was applied by means of a syringe. Rather less solution as compared to ointment was applied.

(b) Plastic film and Blenderm\(^1\) surgical tape were then applied to occlude the treated and control areas. No further attention was given to the areas until samples were taken.

(c) The skin was then washed with 70% alcohol or 0.9% saline to remove excess drug. However, the most effective way to assure that no residual superficially adsorbed drug remained on the skin samples was to follow initial alcohol washing with five stripplings using Scotch tape and a final washing with the 70% alcohol.

(d) The adequacy of the washing was checked by applying the drug as described in paragraph (a) just prior to sampling to the control side also, then immediately washing it off. This procedure saturates the surface and the loosely-knit uppermost portions of the stratum corneum of the control side in the same manner as that of the treated side, the only difference then being the time allowed for the drug to penetrate. Thus any hydrocortisone detected by subsequent assay of the control side must be due to inadequate washing. The method provides a reliable estimate of the extent to which assays of the treated side include superficially adsorbed hydrocortisone. It was shown that there is little residual hydrocortisone.

(e) Dissection of samples was done at two dermatome settings to cut 100 to 150 micron and 200 to 300 micron thick skin slices, respectively. The uniformity and structure of typical epidermal and dermal slices are shown in Fig. 1 - 3. Note that the 150 micron slice is virtually pure epidermis (Fig. 1). The size of the slices removed varied. The usual size was approximately 10 x 12 mm in area and weighed approximately 10 to 20 mg (wet weight) for the thicker slices and 0.5 to 10 mg for the others. Single and sequential serial slices of epidermis and dermis were taken. The removal of single slices is virtually painless and required no anaesthesia. The removal of serial slices required 2% Xylocaine anaesthesia. Contamination by bleeding was not a problem.

(f) The samples were stored on dry ParafilmR in closed Petri dishes which contained wet filter paper. The samples were refrigerated within one to two hours and assayed within 24 hours.
Fig. 1 — Human Epidermis approximately 150 microns thick (0.1 mm setting)

X 90, H. and E. Stain

Fig. 2 — Human Dermis approximately 300 microns thick (0.2 mm setting)

X 90, H. and E. Stain

Fig. 3 — Hairless Mouse Dermis approximately 100 microns thick (0.2 mm setting)

X 90, H. and E. Stain
Preparation of Standards

Standard hydrocortisone solutions were prepared by dilution of a 10 μg/ml stock concentrate in 10% ethyl alcohol, then extracted and analysed as described in the following sections.

For recovery experiments of hydrocortisone in the presence of tissue, internal "hydrocortisone and tissue" standard solutions were prepared from normal epidermis obtained at autopsy from the abdominal area of males and females. These were set up in several series consisting of 5 to 15 mg homogenized epidermal tissue in 1.0 ml 10% ethyl alcohol and 1.0 ml of appropriate hydrocortisone standard solution. There was little analytical interference by tissue. Both hydrocortisone and "hydrocortisone and tissue" standard solution gave straight line curves passing through the origin well within the limits of weighing and measuring experimental errors.

Preparation and Weighing of Samples

Excess moisture was removed from the tissue by blotting it between Whatman #1 filter paper. It was then weighed. Wet weights were determined with a weighing accuracy of ± 0.3 mg for samples greater than 5 mg and of ± 0.03 mg for samples smaller than this. The removal of excess water varied an estimated 10% from sample to sample because of slight differences in pressure exerted during blotting. The weighing was done by the same operator.

The weighed sample was then transferred to the microhomogenizer and homogenized in 0.2 to 0.4 ml 10% ethyl alcohol. The tissue was homogenized until apparently uniform, then quantitatively transferred to extraction tubes with the aid of 10% ethyl alcohol. All samples were prepared for extraction in volumes of 2 ml.
Spectrophotofluorometric Analysis

(a) Two ml of the sample or standard were transferred for extraction to test tubes. 15.0 ml of dichloromethane were added. The solutions were mixed on the Vortex mixer for a few seconds, then placed in a mechanical shaker with the long axis of the tube parallel to the motion of the shaker. In this way, the maximum contact was obtained between the immiscible solvents. The extraction was allowed to proceed for 20 minutes.

(b) The tubes were removed from the shaker, the solutions allowed to separate, and the top aqueous ethyl alcohol layer aspirated with a cotton plugged Pasteur pipette fitted with a rubber bulb.

(c) The solvent layer was filtered rapidly through a dry fluted Whatman #1 filter paper which served to remove the tissue debris and to dry the dichloromethane.

(d) 10.0 ml of this extractive was removed using a cotton plugged pipette fitted with a Propipette. The remainder of the solution was discarded and the aliquot was returned to the collecting tube.

(e) 5.0 ml of Fluorescence Reagent permitted to come to room temperature was placed into each of 2 x 15 cm tubes.

(f) The dichloromethane extractive (paragraph (d)) was added to the fluorescence reagent (paragraph (e)) and shaken in the Vortex mixer for 25 seconds by stopwatch.
(g) The layers were permitted to separate for 3 to 4 minutes, the upper dichloromethane layer poured off, and lower acid layer transferred to quartz cuvettes. Separation by centrifugation was found to be unnecessary. Any residual dichloromethane separates rapidly in the cuvette and does not interfere with fluorescence.

(h) Relative Fluorescence Intensity was read after 10 minutes ± 10 seconds. The excitation wavelength was 470 μm, the emission wavelength 525 μm. Six samples can be readily analysed within seventeen minutes by a single operator.
(i) Calculations

Tissue concentration of hydrocortisone in the samples was expressed as \( \mu g \) hydrocortisone per Gm wet weight of tissue. Example of a typical calculation is given below.

Wet weight treated sample: 5.37 mg
R. F. I. of tissue extractive: 2.43 R. F. I. units
R. F. I. of reagent blank: 0.12 R. F. I. units
R. F. I. of 1.0 \( \mu g \) hydrocortisone standard: 2.46 R. F. I. units

Regression Equation: \[ R.F.I. = 0.00 + 2.32 \times \mu g \text{ hydrocortisone} \]

Check for application of data to regression equation:

\[ R.F.I. \text{ (Regression equation)} = 2.32 \quad R.F.I. \text{ (standard)} = 2.34 \]

\( \mu g \) Hydrocortisone = R.F.I. (blank subtracted)

\[ \frac{2.46 - 0.12}{2.32} = \frac{2.34}{2.32} = 0.996 \]

\( \mu g \) hydrocortisone = 0.996 \( \mu g \) hydrocortisone in the sample

\[ \text{Tissue concentration} = \frac{0.996 \mu g \text{ hydrocortisone} \times 1000 \text{ mg}}{5.37 \text{ mg Gm}} \]

\[ = 185 \mu g \text{ hydrocortisone/Gm tissue (wet weight).} \]
RESULTS AND DISCUSSION

Reliability of Hydrocortisone Assay in Skin

In fluorescence of dilute solutions, the equation comparable to Beer's Law in spectrophotometry is:

\[ F = I \sum E c d \phi \]  \hspace{1cm} (Equation 1)

where \( F \) is the total number of quanta emitted in all directions and at all wavelengths; \( I \) is the number of photons per second incident on the sample; \( E \) is the extinction coefficient, the property determined in a spectrophotometer; \( c \) is the concentration; \( \phi \) is the quantum yield, the probability that having absorbed a photon, the molecule will emit a photon. This may lie between 0 and 1. If \( \phi \) is 0, or nearly so, the compound is nonfluorescent; \( d \), the effective pathlength, is a geometric factor, normally determined empirically.

When a solution is measured with a spectrophotometer, a plot of absorbance as a function of wavelength gives the characteristic absorption spectral curve which is the variation of the extinction coefficient \( E \) with wavelength.

A fluorescent compound is characterized by two spectra. The excitation spectrum is obtained by plotting the intensity of fluorescence as a function of the wavelength of the exciting light, which in the Aminco-Bowman Spectrophotofluorometer is a Xenon source of light, passed through a monochromator. This amounts to holding everything in Equation 1 constant except \( E \). The intensity of the exciting light, \( I_{q} \), is held constant, but since wavelength is being varied, \( E \), the extinction coefficient changes. The excitation spectrum is therefore the absorption spectrum.
The emission spectrum is obtained by fixing the excitation wavelength and plotting fluorescence intensity against emission wavelength. The shape and position of the curve are independent of the wavelength of the exciting light, but the magnitude of the curve will depend on the position in the excitation spectrum.

Fluorescence is emitted uniformly in all directions, making measurement of a portion of the emitted light sufficient. For quantitative and qualitative analysis, the fraction measured must be constant, but need not be accurately known. Since it is not the quanta of light, but an amplified signal from a phototube which is measured, Equation 2 should be re-written as

\[ F_A = K I_q \text{Ecd\(\phi\)} \]  

(Equation 2)

where \( F_A \) is the apparent fluorescence (signal from the instrument); \( K \) is the constant which takes into account the angle of the pickup of light, the electrical amplification, and the response of the phototube to the particular wavelength of light incident upon it. The holding of all instrumental settings constant renders the signal \( F_A \) (expressed in this thesis as R. F. I.) directly proportional to concentration (23).

The intensity of this signal depends upon the nature of the fluorescent substance, the frequency of the incident light, the highest intensity being in the region of maximum absorption, the nature of the solvent, the pH of the medium, the temperature, fluorescence being higher at low temperature, the geometry of the exciting beam within the cell, and the concentration of the fluorescent substance (42).
Excitation and Emission Spectra

Instrument monochromator settings were determined by scanning independently excitation and emission spectra for reagent and tissue (autopsy skin slices) blanks and standards.

Figure 4 shows semi logarithmic graphs of R. F. I. against excitation wavelength for Standard hydrocortisone solution, reagent blanks, and unextracted reagent. All three had peaks coinciding at 470 μm. The similarity of the spectra for reagent blank and reagent not extracted by the solvent indicates the acceptability of the solvent system. The very high peak for hydrocortisone (the peak is actually much higher than a semilog plot indicates at first glance) verifies the measureability of hydrocortisone in contrast to the blank. Figure 5 similarly shows the excitation spectra for tissue standards and tissue blank. The same coincidence of peaks was observed in the presence of either epidermal or dermal skin, indicating that any fluorescent impurities extracted from tissue at any depth of interest do not interfere with the excitation peaks.
Fig. 4 - Hydrocortisone fluorescence excitation spectrum.

1. 1.0 μg/2 ml hydrocortisone in Fluorescence Reagent.

2. Fluorescence Reagent Blank

3. Fluorescence Reagent

Emission Monochromator Setting: 525 μm.
Fig. 5 - Influence of epidermal and dermal tissue extractives on hydrocortisone fluorescence excitation spectrum.

1. ______ 5 mg epidermal tissue extractive and 0.4 μg/2ml hydrocortisone in Fluorescence Reagent

2. ______ 5 mg of dermal tissue extractive and 0.4 μg/2 ml hydrocortisone in Fluorescence Reagent

3. ______ Tissue blank

Emission Monochromator setting: 525 μm.
Fig. 6 and Fig. 7 demonstrate that neither epidermal nor dermal tissue extractives alter the hydrocortisone emission spectrum which shows a peak at 525 μm. Reagent blanks, however, showed an upper plateau from 500 to 525 μm (Fig. 6), while tissue blanks showed a slight but definite broadening and shift of the maximum to 505 to 515 μm (Fig. 7). A reasonable conjecture as to the cause of this may well be that there are substances in skin tissue which themselves show peak fluorescence at this wavelength. This is not the entire answer, however, because tissue blanks show fluorescence intensities of lesser magnitude (at 525 μm) than do reagent blanks. Perhaps tissue contains substances which emulsify or otherwise complex and remove fluorescent impurities in the reagent or solvent. The difference between the two is insignificant, since it shows up only in untreated tissue, and is very slight.

The scatter peaks in all figures are some 50 μm removed from the measuring peaks and provide no interference. By setting to the maxima, excitation 470 μm, emission 525 μm, highly sensitive quantitative analyses can be performed.
Fig. 6 -- Hydrocortisone fluorescence emission spectrum.

1. __________ 1.0 µg/2 ml hydrocortisone in fluorescence reagent
2. ________ Fluorescence Reagent blank
3. ________ Fluorescence Reagent

Excitation Monochromator Setting: 467 µµµ.
Fig. 7 — Influence of epidermal and dermal tissue extractives on hydrocortisone fluorescence emission spectrum.

1. __________ 5 mg epidermal tissue extractive and 0.4 µg/2 ml hydrocortisone in Fluorescence Reagent

2. __________ 5 mg dermal tissue extractive and 0.4 µg/2 ml hydrocortisone in Fluorescence Reagent

3. __________ Tissue blank

Excitation Monochromator setting: 467 µm.
Influence of Time on Fluorescence Development

Ten minutes ± 10 seconds were allowed for fluorescence development in all assays. The timing is critical. When insufficient time is allowed, the fluorescence producing reaction between hydrocortisone and the sulphuric acid - ethyl alcohol Fluorescence Reagent will not have reached a steady state. If too much time is allowed, nonspecific fluorogens from solvents and tissue may also be produced, resulting in false high values or vice versa as the fluorescent species is destroyed. Various time intervals have been reported for hydrocortisone assays in blood (25, 33, 37). This variation is due in part to variation in reagent concentration. It was decided, therefore, to determine more closely the effects of time on fluorescence development.

The R.F.I. was determined for the various components of the hydrocortisone - reagent reaction mixtures separately over a period of 5 to 40 minutes starting from the moment of addition of solvent to reagent (Fig. 8). Hydrocortisone standards without tissue were similar to those with tissue, while tissue blanks were similar to the reagent blank. Hydrocortisone shows a rapidly developing intense fluorescence which begins to level off at about 10 minutes. Blanks show a gradual development of slight fluorescence which also levels off at about 10 minutes. This levelling off was independent of the nature of solvents, the presence of tissue or of the concentration of hydrocortisone. The initial rise indicates that some nonspecific fluorogens are produced by sulphuric acid interactions with the small amounts of dichloromethane impurities and tissue extractives carried over into the final reaction mixture. The level of fluorescence produced by the nonspecific fluorogens was low compared to that of hydrocortisone (Fig. 6 & 7) and did not interfere with the assay.
Fig. 8 — Influence of time on the development of fluorescence.

1. Reagent Blank (2 ml of 10% ethyl alcohol carried through the extraction)
2. 1.0 ug Hydrocortisone standard solution in the presence of tissue.
The validity of the choice of 10 minutes for fluorescence development time is also shown by the data in Table I. Relative fluorescence intensity was determined at 5, 10 and 13 minute time intervals for 200 to 300 micron skin slices from eight patients who had absorbed varying amounts of hydrocortisone in epidermis. The table shows that maximum R.F.I. is reached in about 10 minutes. The proportionality of the rates of fluorescence maintained over a tenfold range of hydrocortisone concentration is also shown. At very low R.F.I., due to low amounts of hydrocortisone, the maximum appears to be reached sooner, i.e., in 5 minutes. Ten and thirteen minute fluorescence development times give virtually the same values for hydrocortisone concentration.

TABLE I

<table>
<thead>
<tr>
<th>Hydrocortisone, in µg per Gm wet weight of epidermis</th>
<th>5 Minute Readings</th>
<th>10 Minute Readings</th>
<th>13 Minute Readings</th>
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</thead>
<tbody>
<tr>
<td>260</td>
<td>303</td>
<td>308</td>
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</tr>
<tr>
<td>6</td>
<td>16</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

a Assayed in epidermis after absorption from 2.5% hydrocortisone in Hydrophilic Ointment. R.F.I. values were converted to µg hydrocortisone from the same standards assayed at each of the three time intervals, 5, 10, and 13 minutes, respectively.
Standard Curves

The standard curves shown in Fig. 9 and Fig. 10 verify the linear relationship between R. F. I. and the concentration of 0.1 to 20 μg hydrocortisone dissolved in 2 ml of 10% ethyl alcohol. The amount of hydrocortisone absorbed rarely exceeded 2 μg per sample for even 20 mg samples. This was fortunate because it permitted the use of one standard curve (Fig. 9) for all calculations. The effect of interfering fluorescence from tissues on the standard curve was determined by homogenizing 10 mg of human epidermal and dermal autopsy tissue along with various concentrations of hydrocortisone up to a concentration of 2.5 μg hydrocortisone per sample. Fig. 9 shows that the R. F. I. of the hydrocortisone recovered from the presence of the autopsy tissue had the same magnitude as when the drug alone was assayed. No evidence of fluorescence quenching by increasing hydrocortisone concentrations was observed. This is in conformity with the experience of Gantt (24) who found linear R. F. I. - concentration relationships for hydrocortisone assaying as high as 40 μg per sample. Within the range investigated, there were no quenching effects due to the presence of tissue.

The regression equation for the 0.1 to 2.5 μg range was:

\[ R. \ F. \ I. = 0.00 + 2.32(μg \ hydrocortisone) \]

in the presence and absence of tissue, and for the entire 0.1 to 20 μg range:

\[ R. \ F. \ I. = 0.15 + (2.48) (μg \ hydrocortisone) \]

in the absence of tissue.

The linearity of the standard curve and its passage exactly or nearly through the origin is strong, although not absolute, evidence for the validity of the assay.
Fig. 9 -- Standard curve of hydrocortisone with and without the presence of skin tissue (low concentration range).

- - - - - - Hydrocortisone in 10% ethyl alcohol

0 - - - - - - Hydrocortisone and 10 mg human autopsy tissue in 10% ethyl alcohol.
Fig. 10 -- Standard curve of hydrocortisone (high concentration range).
Interference by Foreign Substances

An analytical technique is only as useful as its breadth of application. A variety of vehicles and drugs may be present in topical preparations of hydrocortisone. The interference by some of these is considered in this section. The most important potential contaminants for the purposes of this study were the components of Hydrophilic Ointment, U.S.P. XVII, of the local anaesthetic, Xylocaine 2% and Epinephrine 1:100,000, and of the dermatome lubricating oil, Tellus No. 33 Lubricating Oil. The extent of their interference was ascertained by assaying excess amounts of each. Hydrophilic Ointment showed a R.F.I. equivalent to less than 10 μg hydrocortisone per Gm of base (mg quantities are applied to the skin). Xylocaine solution showed a R.F.I. equivalent to 3 μg hydrocortisone per Gm of solution. Extraction of several drops of dermatome oil was analytically indistinguishable from the reagent blank. Table II shows that the fluorescence produced by these substances can be neglected because it does not exceed variations encountered in assays of untreated skin.

TABLE II

FLUORESCENCE PRODUCED BY TOPICAL ADMINISTRATION OF HYDROPHILIC OINTMENT AND LOCAL ANAESTHETIC

<table>
<thead>
<tr>
<th>Hydrocortisone in μg per Gm wet weight of epidermis</th>
<th>Untreated Skin</th>
<th>Hydrophilic Ointment Treated Skin</th>
<th>Hydrophilic Ointment and Xylocaine Intradermal anaesthesia treated skin</th>
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<td>9</td>
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<tr>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

a Expressed as hydrocortisone equivalent.

1 Storz Instrument Co., St. Louis, Missouri.
Neomycin sulphate and lanolin derivatives are frequently incorporated in commercial hydrocortisone preparations. Neomycin sulphate produced no detectable fluorescence, possibly because the salt is not extracted by the nonpolar dichloromethane. An ointment base was prepared which consisted of substituted lanolin derivatives, Acetulan$^R$ and Solulan$^R$. The base has been recommended by the manufacturer as enhancing steroid penetration in skin$^1$. The base showed a R.F.I. equivalent to 100 μg hydrocortisone per Gm of base. However, when applied in the usual mg quantities to skin, no interference was observed. The indications are, therefore, strong that the proposed hydrocortisone assay may well find wide use for the evaluation of relative penetration rates of the steroid from many topical dosage forms.

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1 American Cholesterol Products Inc., Edison, New Jersey. Personal Communication to Dr. J.O. Runikis.
Reproducibility and Error Estimation

The experimental error of the assay may be regarded as arising from three sources: determinate sources of errors, indeterminate sources of errors, and errors arising from the need to express the concentration of hydrocortisone per unit wet weight of tissue. These are discussed below in sections (a), (b), and (c).

(a) Errors arising from determinate sources of errors such as from non-specific reactions producing fluorogens other than those due to hydrocortisone, incompleteness of homogenization and extraction procedures, instrument optics and electronics and other similar sources of error which influence the accuracy of the assay.

No quantitative evaluation of the accuracy of the method has been done. The indirect evidence, described in previous sections of this thesis, indicates that the proposed assay is sufficiently accurate for the determination of amounts as low as 0.1 µg of hydrocortisone in skin. The average recovery of hydrocortisone in the presence of tissue is approximately 10 to 15% higher than the theoretical, (See Table III). The one naturally occurring substance which may invalidate the assay is corticosterone (56). As shown by numerous assays of control skin samples, neither corticosterone nor endogenous hydrocortisone exist in appreciable quantities in skin. The fact that the blood concentrations of hydrocortisone are very low also tends to support these observations. However, the possibility may be argued that the low endogenous hydrocortisone levels are observed only because hydrocortisone is strongly adsorbed to cell membranes or other structures and is not freed by the assay. Applied hydrocortisone may similarly be bound. It is suggested that this possibility
be checked through the use of more complete homogenization procedures, such as ultrasonic homogenization, since the results would also help to prove or disprove the view held by some that hydrocortisone is a metabolite occurring in all organs.

(b) Errors arising from indeterminate sources of errors, some examples of which are variations in measurement and sample manipulation technique, indeterminate fluctuations of the electronic components of the instrument and variations in the histochemical composition of the skin samples. To test the reproducibility of the assay and % recovery of hydrocortisone in the presence of skin tissue, three series of hydrocortisone solutions were assayed. These samples consisted of twelve 2ml samples of 1 ug hydrocortisone standard solutions, 1 ug hydrocortisone standard solutions plus 5 mg of human autopsy epidermal tissue, and 1 ug hydrocortisone standard solution plus 5 mg human autopsy dermal tissue. The reproducibility is expressed in terms of range, the standard deviation and the standard error of the mean in Table III.

<table>
<thead>
<tr>
<th>Sample</th>
<th># of Observations</th>
<th>Hydrocortisone Recovered</th>
<th>Range in ug/2ml</th>
<th>Standard Deviation in ug/2ml</th>
<th>Standard Error in ug/2ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000 ug Hydrocortisone</td>
<td>12</td>
<td>1.000</td>
<td>0.948-1.059</td>
<td>0.018</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(theoretical)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.000 ug Hydrocortisone + 5mg epidermis</td>
<td>11</td>
<td>1.136</td>
<td>1.1059-1.190</td>
<td>0.037</td>
<td>0.011</td>
</tr>
<tr>
<td>1.000 ug Hydrocortisone + 5mg dermis</td>
<td>12</td>
<td>1.125</td>
<td>1.087-1.182</td>
<td>0.030</td>
<td>0.009</td>
</tr>
</tbody>
</table>
The reproducibility with a standard deviation of 2% to 4%, depending on whether tissue is present or not, is very satisfactory for a biological microanalysis. A comparison of the theoretical and actual values for the % of hydrocortisone recovered, a measure of the accuracy of the assay, shows rather higher values than those encountered in measurements of standards plotted in Fig. 9. This may be due to differences in storage. Frozen autopsy tissue was employed for the reproducibility experiments. Fresh autopsy tissue was used for the standard curve of Fig. 9. It appears safe to say, however, that the assay is capable of determining the amount of hydrocortisone in skin with an error of less than 15%.

The reproducibility of the assay was verified also by asking ten student analysts to determine hydrocortisone standard curves, eight of whom in pairs performed the assay only once. The standard curves obtained by all participants were essentially the same.

(c) Errors arising from uncertainties in expressing the concentration of hydrocortisone per unit weight of tissue. These in turn arise because of faulty histological localization of the steroid in skin due to incomplete removal of residual hydrocortisone from the skin surface, the gross mechanical dissection procedure used, and the uncertainties inherent in determining 'true' tissue weight.

The errors due to incomplete removal of residual hydrocortisone were eliminated by the washing-stripping-washing procedure described in a previous section, but possibly at the cost of creating a new source of error. It has been suggested by Vickers and others (2) that stripping may damage the layers of the stratum conjunctum bordering the stratum
granulosum which is the site of hydrocortisone depot formation (19).
This source of error was not thoroughly investigated. However, random
histological slides showed no damage to the stratum granulosum.

The samples designated as 'epidermis' in this thesis had loose sur-
face layers of the stratum corneum removed but contained varying amounts
of dermis. The dermal contamination produced no positive errors since,
as is further demonstrated, the dermis retains virtually no hydrocortisone.
However, this contamination may produce negative errors if the dermis con-
tributes substantially to the weight, i.e., dilution effect. Conversely,
the samples designated as 'dermis' may have included traces of epidermal
tissue which stores substantial amounts of hydrocortisone rendering a
positive error. The precaution was taken to cut dermal slices with a smal-
ler blade width than the overlying epidermal layers. Random histological
slides showed no epidermal contamination.

The near impossibility of determining representative tissue weight
because of differences in histological composition and moisture content
between the skin slices may give rise to erroneous comparisons between
patients. The tissue weight can be expressed as dry weights or as wet
weights. The dry weights yield more reliable comparisons but only if
the dry weight of each sample is determined separately. This was found
impractical for the purposes of this study. The wet weight / dry weight
ratio, however, was determined for a series of 10 washed and stripped
epidermal slices of human autopsy tissue. The wet weight / dry weight
ratio was 2.23 with the rather large standard deviation of 0.18. The tissue concentration is reported throughout the thesis in terms of μg hydrocortisone per Gm of wet weight. It is difficult to give a firm figure for the uncertainties in the determination of tissue weights. It is believed that on occasion, an error as large as 20% may be present. Lyophilization would eliminate this problem.
Applicability of the Assay to Other Corticosteroids

Hydrocortisone Sodium succinate was measured in skin only and its concentration expressed in terms of hydrocortisone alcohol. Although standards were not prepared, this form of the drug would appear to lend itself well to rigorous assay.

A preliminary screening has been done to ascertain the possibility of utilizing the assay for the analysis of corticosteroids other than hydrocortisone. The results are listed in Table IV. The assay procedure was left essentially unmodified, except for the changes indicated. No appreciable chemical shifts in fluorescence emission spectra were observed. The wavelength settings for all of the corticosteroids, except triamcinolone acetonide, were 467 μm for excitation and 525 μm for emission. Pure chemicals only were used.
TABLE IV

<table>
<thead>
<tr>
<th>Name</th>
<th>Modifications in Assay Procedure</th>
<th>Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triamcinolone Acetonide</td>
<td>Excitation wavelength: 467 μm, emission wavelength 510 μm.</td>
<td>Negligible fluorescence even for large samples.</td>
</tr>
<tr>
<td>Triamcinolone Acetonide</td>
<td>Chemical added directly to Fluorescence Reagent</td>
<td>Negligible fluorescence</td>
</tr>
<tr>
<td>Fluocinolone Acetonide</td>
<td>No modification</td>
<td>Negligible fluorescence</td>
</tr>
<tr>
<td>Fluocinolone Acetonide</td>
<td>Chemical added directly to Fluorescence Reagent</td>
<td>Negligible Fluorescence</td>
</tr>
<tr>
<td>Betamethasone-17-valerate</td>
<td>No modification</td>
<td>Negligible fluorescence</td>
</tr>
<tr>
<td>Betamethasone-17-valerate</td>
<td>Chemical added directly to Fluorescence Reagent 20 minute readings</td>
<td>Intense fluorescence, further study warranted</td>
</tr>
<tr>
<td>Dichlorisone Acetate</td>
<td>Insoluble in 10% Ethyl Alcohol and dichloromethane</td>
<td>-</td>
</tr>
<tr>
<td>Dichlorisone Acetate</td>
<td>Chemical added directly to fluorescence reagent</td>
<td>Intense fluorescence, further study warranted</td>
</tr>
</tbody>
</table>

The proposed assay, therefore, appears to be quite specific and its present form applicable to hydrocortisone only. There can be little doubt, however, that a spectrophotofluorometric assay along the lines suggested in this thesis, applicable to drug penetration studies in skin, could be developed also for other corticosteroids. Under the right conditions of pH, fluorescence development time, solvent system, etc., all compounds can be made to fluoresce. Partial support for this statement can be derived from the observations made with betamethasone-17-valerate and dichlorisone acetate, the former of which contains all of the chemical groups apparently required for adequate fluorescence, the latter of which replaces the 'required' oxygen moiety with a chlorine atom. Replacement of dichloromethane by some other selective extracting solvent, a tedious but not insurmountable problem, would almost certainly lead to a fluorescence assay procedure for these corticosteroids.
Hydrocortisone in Human Epidermis and Dermis

The concluding part of this thesis demonstrates the validity of the combination of the dermatome technique and the spectrophotofluorometric assay as a suitable method for the study of hydrocortisone penetration rates through skin in vivo. A later section presents preliminary evidence that the method may be readily adapted also to clinical pharmacological studies of drug activity within the skin.

Hydrocortisone Absorption in Epidermis

Table V is presented to show that substantial amounts of hydrocortisone are recovered only from skin sites treated with drug. Untreated skin sites, as expected, show little or no hydrocortisone. 2.5% hydrocortisone was applied to hospitalized patients with normal skin. Paired experiments were done. The vehicle was applied to the control site under occlusive dressing. Duration and manner of administration varied, but was the same for the treated sites and their controls. Before dissections, 2.5% hydrocortisone was applied for a brief moment to the control sites of the 0.1 mm samples to test for the efficacy of the washing procedure as previously described. Exceptions are noted. 0.1 mm slices were washed with 70% alcohol. 0.2 mm slices were washed with 0.9% saline. The epidermal slices were removed at these two (0.1 and 0.2 mm) depth settings of the dermatome. The thicker slices obtained at the 0.2 mm dermatome settings had correspondingly more dermal tissue. The actual thickness of the slice may be up to 50% more than indicated by the nominal value. The weights are expressed on wet weight basis.
<table>
<thead>
<tr>
<th>Patient NUMBER</th>
<th>Treated Site</th>
<th>Sample Weight (mg)</th>
<th>Hydrocortisone Concentration (ug/Gm tissue)</th>
<th>Control Site</th>
<th>Sample Weight (mg)</th>
<th>Hydrocortisone Concentration (ug/Gm tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1 mm slices</td>
<td>4.8</td>
<td>358</td>
<td>6.8</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>4.9</td>
<td>169</td>
<td>3.3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3Da</td>
<td></td>
<td>0.67</td>
<td>98</td>
<td>3.02</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>3D</td>
<td></td>
<td>-</td>
<td>-</td>
<td>5.15</td>
<td>30.0b</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>65</td>
<td></td>
<td>11.6</td>
<td>0b</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.33</td>
<td>33</td>
<td></td>
<td>4.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3.17</td>
<td>30</td>
<td></td>
<td>4.04</td>
<td>0b</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.9</td>
<td>21</td>
<td></td>
<td>11.6</td>
<td>0b</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.2 mm slices</td>
<td>10.4</td>
<td>335</td>
<td>7.3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>9D</td>
<td></td>
<td>12.1</td>
<td>131</td>
<td>15.8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9D</td>
<td></td>
<td>11.9</td>
<td>131</td>
<td>9.6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>10c</td>
<td></td>
<td>4.8</td>
<td>130</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>11D</td>
<td></td>
<td>20.3</td>
<td>111</td>
<td>17.2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>11D</td>
<td></td>
<td>18.6</td>
<td>99</td>
<td>18.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12D</td>
<td></td>
<td>8.2</td>
<td>66</td>
<td>7.7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>12D</td>
<td></td>
<td>-</td>
<td>-</td>
<td>7.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13Dd</td>
<td></td>
<td>15.1</td>
<td>47</td>
<td>9.1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>13D</td>
<td></td>
<td>15.3</td>
<td>35</td>
<td>9.2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>20.7</td>
<td>20</td>
<td>11.1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

a The letter D serves to emphasize duplicate samples removed from the same patient.

b Effectiveness of washing not checked by the reapplication of hydrocortisone.

c Solution B solution was applied; no hydrophilic ointment.

d Non-occlusive dressing.
It can be concluded that treated areas only show significant amounts of hydrocortisone absorbed. Two patients showed relatively large amounts of hydrocortisone in control sites. The high values for these patients are possibly explained below (Table VI). Duplicate samples from the same patient are in excellent agreement, both for large and small concentrations of hydrocortisone. Sample size between 0.5 to 20 mg does not affect the assay.

The accumulation of hydrocortisone in epidermis varies greatly. These differences may be due to one or more of several causes. Some of these are the manner of administration, e.g., whether occlusive or not, the time interval elapsed between administration and sample removal, biological variations in histological structure and in susceptibility to drug action such as the vasoconstriction effect described subsequently. Initial attempts to resolve the causes have been made.

Effects due to surface contamination were ruled out as the procedure was improved. The certainty of freedom from surface contamination can be achieved only upon stripping the skin with Scotch tape (five times) in between two washes with 70% alcohol. The necessity for the stripping is emphasized by the following typical data which compares three washing procedures. 2.5% hydrocortisone was applied from the hydrophilic ointment base. The hydrocortisone did not penetrate the skin.

<table>
<thead>
<tr>
<th>Hydrocortisone residue µg/Gm tissue</th>
<th>Washing Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>Single 70% alcohol wash</td>
</tr>
<tr>
<td>30</td>
<td>Single 70% alcohol wash and stripping</td>
</tr>
<tr>
<td>0</td>
<td>Stripping in between 70% alcohol washes.</td>
</tr>
</tbody>
</table>
Influence of Time on Hydrocortisone Absorption

Regrouping the data in Table V brings out the effects of the duration of hydrocortisone contact upon the accumulation of the steroid in the epidermis (Table VII). Application was under occlusive dressing. The data will also illustrate the applicability of the method to kinetic studies.

### TABLE VII

**EFFECT OF TIME OF CONTACT ON THE ACCUMULATION OF HYDROCORTISONE IN EPIDERMIS**

<table>
<thead>
<tr>
<th>ug Hydrocortisone per Gm Wet Weight of Epidermis</th>
<th>4 hours</th>
<th>6 hours</th>
<th>12 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hours</td>
<td>66</td>
<td>358</td>
<td>335</td>
</tr>
<tr>
<td>4 hours</td>
<td>20</td>
<td>169</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>130a</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average:</td>
<td>43</td>
<td>118</td>
<td>175</td>
</tr>
<tr>
<td>(2 observations)</td>
<td>(6 observations)</td>
<td>(4 observations)</td>
<td></td>
</tr>
</tbody>
</table>

*a Solution of Solu-Cortef*

The data represent different patients. The 6 hour data are from 0.1 mm slices. The others are from 0.2 mm slices. The 4 and 12 hour concentrations are low by a factor as much as 2 because they contain a relatively higher weight proportion of inert dermis which does not store hydrocortisone.

The number of observations is insufficient. However, the following trends should be noted for their value as clues for further investigations. Hydrocortisone continues to accumulate throughout the 12 hour period investigated. There are large individual variations between the amount of hydrocortisone stored in the epidermis during the first 6 hours.
These differences in the size of the depot formed in the epidermis appear to level off at 12 hours. If these trends were confirmed, they might provide rational means to control the amount of the drug maintained in the skin. This is important not only for maintenance of therapeutic dosage levels, but also for reducing systemic drug levels.

The regrouping of Table VII together with the tissue weights listed in Table V illustrates the unique applicability of the method to kinetic studies. Small samples may be assayed readily. Several such small samples of uniform thickness are readily taken by simply narrowing the blade of the dermatome, and should be as acceptable to patients as one or two large slices. Because drug is measured locally only, true bilateral controls for each patient at each stage of several time samples are possible. No such controls are possible with blood sampling after the first application of the drug. Therefore, with this method, a drug may be applied to a series of patients under well-controlled conditions and the accumulation and elimination patterns studied. Immediate application of this would be an attempt to verify the radioautographic work of Scott and Kalz (11) who noted basal layer concentration of hydrocortisone within two hours, presence of drug in cells about blood vessels within 6 hours, and complete disappearance from the skin within 16 hours.

Of course, kinetic studies are limited not only to accumulation of the drug per se but also are of utmost importance in evaluating other parameters such as vehicles, discussed previously, state of the skin, anatomical location, etc., where true controls for each individual patient become mandatory.
Relative Hydrocortisone Absorption in Epidermis and Dermis

A feature of the method is the opportunity it affords for following the penetration of hydrocortisone from epidermis into the dermis by means of serial sequential slices. For the data of Table VIII, a dermal slice was removed in addition to an epidermal slice. Both slices were approximately 100 to 150 microns thick (0.1 mm dermatome setting). As before, in vivo human skin was used. Intradermal 2% Xylocaine anaesthesia was necessary. The removal of the dermal slice is more complicated because the surface has to be dry before the dermatome cuts can be made. Freezing of the dermal wound with Freez-o-derm Aerosol was sometimes necessary to effect drying in a reasonable time. The 2.5% hydrocortisone ointment was applied under occlusive dressing for six hours.

**TABLE VIII**

**HYDROCORTISONE IN EPIDERMIS AND DERMIS AFTER 6 HOUR ADMINISTRATION**

<table>
<thead>
<tr>
<th>µg Hydrocortisone per Gm Tissue Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated epidermis</td>
</tr>
<tr>
<td>169</td>
</tr>
<tr>
<td>98</td>
</tr>
<tr>
<td>65</td>
</tr>
<tr>
<td>33</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>21</td>
</tr>
</tbody>
</table>

The results are revealing. Essentially no hydrocortisone was detected in the dermis although substantial hydrocortisone depots were present at the same time in the epidermis. These results are significant in spite of the small numbers of observations because the difference between the epidermal and dermal concentrations is so great. They are also in accord
with the findings of other investigators. Vickers and other workers (11) have pinpointed the location of a hydrocortisone depot in the epidermis. Malkinson (4) proposes that after the passage through the epidermis there is no more significant hindrance in the corium for drug entry into the circulation via the capillaries. The hydrocortisone depot in the epidermis should maintain an equilibrium concentration in the dermis. Assuming no adsorption of hydrocortisone in the dermis, this equilibrium concentration should be expected to be below the sensitivity of the fluorescence assay since the solubility of hydrocortisone is small and since the depots are of microgram size. It should be noted in passing that those samples listed as showing no hydrocortisone present frequently showed a fluorescence intensity lower than the reagent blank. This is attributed to the shift of peak fluorescence emission for tissue blanks (Fig. 8), previously discussed. Acceptance of the levels as 0 is believed to be valid.
Influence of Hydrocortisone on Skin Functions

The greatest usefulness of the method is envisaged in its applications to in situ studies of drug effects on skin functions. To test this aspect of the method, a few analytical procedures were carried out in conjunction with epidermal respiration rate measurements and deoxyribonucleic acid content (DNA) determinations. Part of the skin slice (0.2 mm dermatome setting) was used for analysis and another part for the other determinations. Observations were also done to record the presence or the absence of blanching due to the vasoconstriction response of skin to hydrocortisone applications (18). Table IX lists the results. Epidermal respiration is expressed in μl oxygen consumed per μg DNA per hour (QO₂). DNA content was chosen as a constant parameter permitting comparisons of respiration rates in terms of the number of metabolically active cells present in the skin slice (57). The measurements were done at various time intervals.

<table>
<thead>
<tr>
<th>Hydrocortisone Depot</th>
<th>Epidermal Respiration Ratio QO₂(DNA) Control Side</th>
<th>Vasoconstriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg/Gm epidermis</td>
<td>Control Side</td>
<td>Treated Side</td>
</tr>
<tr>
<td>335</td>
<td>1.47</td>
<td>1.46</td>
</tr>
<tr>
<td>131</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>105</td>
<td>1.43</td>
<td>1.43</td>
</tr>
<tr>
<td>66</td>
<td>1.88</td>
<td>1.88</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>358</td>
<td></td>
<td></td>
</tr>
<tr>
<td>169</td>
<td></td>
<td></td>
</tr>
<tr>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
These results are of importance only in so far as they demonstrate that drug concentration can be determined in actively metabolizing skin and that simultaneous correlations between measurements of skin function and intracutaneous drug concentration are possible. It might be noted, however, that there appears to be no correlation between the amount of hydrocortisone in the depot and the skin respiration rates. All respiration rates were depressed. The vasoconstriction does, however, appear to correlate with the amount of hydrocortisone in the epidermis. Vasoconstriction was absent for hydrocortisone concentration below 65 μg per Gm epidermis, except for the same atypical sample which showed both greatly depressed respiration rates and vasoconstriction but low concentrations of hydrocortisone in the depot. It may be that insufficient time was allowed for vasoconstriction to appear.¹

¹ Stoughton, R.B., Personal communication.
SUMMARY

1. A method for the assessment of drug activity in skin by quantitative drug penetration measurements has been described. Hydrocortisone, a representative anti-inflammatory steroid of clinical and pharmaceutical interest, was the drug selected to develop the method. It was administered mainly in the form of a 2.5% ointment under occlusive dressings to volunteer patients.

2. The depth of penetration was followed by a new dermatome serial slicing technique. Uniform epidermal and dermal slices of 100 to 150 μ in thickness were removed in vivo from human and animal skin. No anaesthesia was required for the dissection of epidermal slices. Intradermal 2% Xylocaine anesthesia with and without 1:100,000 Epinephrine was used for dermal dissections.

3. The samples, 0.5 to 20 mg in size were assayed by a new spectro-photofluorometric technique developed from a well-established technique for blood. The samples were removed 4, 6 and 12 hours after the administration of the hydrocortisone.

4. The assay was done at 470 μ and 525 μ excitation and emission wavelengths, respectively. Hydrocortisone was extracted from homogenized tissue with dichloromethane. Fluorescence was developed with a 85:15 w/w sulphuric acid - ethyl alcohol solution. Ten minutes was chosen for fluorescence development.

5. The analytical reliability was ascertained as follows. Hydrocortisone excitation and emission spectra were determined in the presence and absence of skin tissue. No chemical shifts were detected. The scatter peaks provide no interference. Standard curves were linear and passed
through the origin. Vehicle ingredients, Xylocaine, Epinephrine and other potential contaminants of relevance to this investigation showed no significant interfering fluorescence. The % recovery of hydrocortisone homogenized together with epidermal and dermal tissue was 114% and 112%, respectively, a small discrepancy for this type of assay. The standard error in three series of 12 samples was 0.005 ug for hydrocortisone assayed from standard solutions, 0.011 ug for hydrocortisone assayed from homogenates of epidermal tissue and 0.009 ug for hydrocortisone assayed from homogenates of dermal tissue, with a theoretical mean of 1.000 ug.

6. The validity of the method for drug penetration studies was ascertained as follows. A special procedure was developed to assure complete removal of ointment residues from the skin surface. A series of 14 bilaterally symmetrical paired measurements detected hydrocortisone in treated sites only while little or no hydrocortisone was detected in control sites. Simultaneous determinations of the depth of the penetrations of hydrocortisone in epidermis and dermis demonstrated appreciable hydrocortisone depots in the epidermis but none in the dermis, in conformity with theory.

7. The concentration of hydrocortisone in epidermis varied greatly among the 14 patients examined. The range was from 20 ug to 358 ug per Gm wet weight of the tissue. The differences appeared to depend on the manner and time of administration although individual variations in rates of hydrocortisone accumulation also seemed to be encountered. Further work to elucidate the cause of these variations is suggested.

8. Exploratory correlations between skin respiration rates, vaso-constriction, and the amount of hydrocortisone in epidermis have been done. In the insufficiently small number of samples examined, respiration rates were uniformly depressed regardless of the size of the hydro-
cortisone depot in the epidermis. Vasoconstriction was generally absent for hydrocortisone depots below 65 ug per Gm epidermis, but present for larger depots.

9. Experimentation has been initiated to broaden the applicability of the method to other corticosteroids and commercial preparations of hydrocortisone. The results for four halogenated corticosteroids and several ingredients of commercial vehicles have been described.
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