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Skin Lipids. I. Sampling Problems of the Skin and Its Appendages

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Abstract

A review of the problems of sampling various skin tissues for study of lipids is presented following a survey of the anatomy and physiology of skin and its appendages.

Anatomy and Physiology of the Skin (1,2)

THE SKIN SERVES a variety of functions which include regulation of body temperature, sensory contact to the external environment, and production of Vitamin D. The primary function of the skin, however, is that of the protection of the body from the external physical and chemical environment. This is accomplished by the continuous proliferation of living epidermal cells whose ultimate fate is to die and become the outermost horny layer (stratum corneum, keratin) (3).

The skin has three layers: the epidermis, the dermis, and the subcutaneous or adipose tissues (Fig. 1). Overlying the subcutaneous tissue is the dermis (corium) which functions as the physical and nutritional support of the epidermis. The dermis consists mainly of connective tissue fibers (collagen, reticulum and elastin) between which pass the blood vessels and nerves of the skin. The ground substance, an amorphous, viscous, semisolid gel, fills the remaining dermal spaces between the fibers, vessels and nerves. Fibroblasts, the precursors of collagen fibers, are present as well as wandering cells of the blood and reticuloendothelial system.

The epidermis is composed of four layers: the basal or germinative layer, the prickle or Malpighian layer, the granular layer and the outermost horny layer (Fig. 2). Fine nerve fibers terminate in the lower epidermis. No blood vessels, however, are found in epidermis which is nourished by diffusion of nutrients from the underlying dermis. The epidermis varies in thickness from 0.1 to 0.3 mm. That portion of the epidermis which extends from the basal layer through the granular layer is considered as the *living epidermis*, and the outermost horny layer of keratin, the *dead epidermis*.

As the epidermal cells move upward in a pattern of continuous regeneration and enter the upper Malpighian layer, they undergo a process of enzymatic dissolution, become dehydrated and flattened to form the dead, horny layer. The lower horny layer is compact and tightly adherent, while in the upper horny layer, the dead cells lose this adherence and slough as microscopically fine scales. In normal human skin, 27 days are required for the transition from the basal layer to shedding stratum corneum.

The epidermal appendages are three in number: the pilosebaceous apparatus, the eccrine sweat gland, and the apocrine sweat gland. The pilosebaceous apparatus consists of the hair follicle and the multilobulated sebaceous gland (4). The sebaceous gland is connected by a short duct to the follicular canal about midway along the follicle (Figs. 1 and 3). The sebaceous gland cells mature as they grow from the periphery toward the center of the gland, become fat-laden, and disintegrate in the central portion of the gland as is characteristic of holocrine excretion. This is not a secretory function. This mass of lipid and cellular debris discharges into the sebaceous duct and is called sebum. Sebaceous glands have a variable distribution over the surface of the body. In the human being, they are most numerous on the scalp and forehead, and occur with decreasing frequency on the chest, back, axillae, genitocrural area, abdomen, arm and leg. They are not found on the palms or soles.

Variants of the sebaceous gland exist in man. Large sebaceous glands (often on the nose and cheeks) may be associated with tiny vellus hairs, the size of the gland being inversely proportional to the size of the associated hair structure. These glands, with large, dilated, patulous ducts and rudimentary hairs, have been called sebaceous follicles (5). Sebaceous glands may occur independent of hairs and excrete directly onto the skin surface. In the human eyelid, Meibomian glands, a modified sebaceous gland, empty their contents at the margin of the eyelids. The cerumenous glands of the human external ear canal are also modified sebaceous glands. Typical sebaceous glands are also found in the external ear canal as well as eccrine and apocrine sweat glands. Thus, cerumen, or ear

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TABLE I

Source and Other Data on Various Skin Lipid Samples

Sample	Species & anatomical site	Tissue removed	Disease	Fully keratinized cells present	Incompletely keratinized cells present	"Sebum" present	Bacteria present	Other tissue present	Manner lipids extracted
Epidermal									
No. 1 Total epidermis	Human leg	Yes	Arterio-sclerosis	Yes	Yes	Yes	Yes		C/M[a] 2/1
No. 2 Total epidermis	Human leg	Yes	Diabetes	Yes	Yes	Yes	Yes		C/M 2/1
No. 3 Total epidermis	Human soles	Yes	Chronic osteo-myelitis	Yes	Yes	No	Yes		C/M 2/1
No. 4 Stratum corneum	Human leg	Yes	Diabetes	Yes	No	Yes	Yes		C/M 2/1
No. 5 "Living" epidermis	Human leg	Yes	Diabetes	No	Yes	No	No		C/M 2/1
No. 6 Total epidermis	Rat body	Yes		Yes	Yes	Yes	Yes		C/M 2/1
Sebaceous									
No. 7 Sebaceous gland	Human scalp	Yes		No	No	Yes	Yes	Gland sac & minimal duct	C/M 2/1
No. 8 Meibomian glands	Human eyelid	No	None	No	No	Yes	Yes		C/M 2/1
No. 9 Preputial gland	Rat genital	Yes	None	No	No	Yes	Yes	Gland sac & duct	C/M 2/1
No. 10 Preen gland	Goose tail	Yes	None	No	No	Yes	Yes	Gland sac & duct	C/M 2/1
Skin Surface									
No. 11 Human surface	Scalp	No	None	Yes	No	Yes	Yes		Ether soak
No. 12 *Vernix caseosa*	Human body	Yes	None	Yes	No	Yes	No	Lanugo hairs & keratinized cells of fetal skin	C/M 2/1
No. 13 Rat surface	Back	No	None	Yes	No	Yes	Yes		Hexane soaked cotton wipings

[a] C/M = Chloroform/Methanol

wax, represents a composite from these four types of glandular structures.

Sebaceous glands are found in the skin of most mammals with the notable exception of whales and porpoises. In some mammals, sebaceous glands are distributed diffusely in the skin, and in others, they lie grouped in localized areas. Several mammals possess localized sebaceous structures of huge size, such as the rat preputial gland, the inguinal glands of the rabbit, and the brachial glands of both the ring-tailed lemur and the gentle lemur (6).

The second appendage of human epidermis is the eccrine sweat gland whose function is the production of water for evaporative heat loss. These glands are coiled tubular structures, situated deep in the dermis. The ducts pass upward through the dermis and spiral through the epidermis to the skin surface (Fig. 1). These glands have a wide and uniform distribution over the human body including the palms and soles. Lipid granules have been demonstrated in the cells of the eccrine sweat glands by histochemical staining (7). These lipids, however, have not been isolated or examined.

The third appendage, the apocrine sweat gland, found chiefly in the axilla, genitocrural areas, areaola and nipple of the breast, eyelids and external auditory canal, represents an active glandular system whose functions are not yet resolved (8). The apocrine gland secretes a milky viscid fluid which contains lipids.

The skin demonstrates great variability in the number and size of the appendages and in the thickness of the various skin layers from one anatomical site to another. For example, palms and soles have a thick epidermis, predominantly stratum corneum, but lack sebaceous glands and hair (Fig. 4). Anatomical variations at a given site in the skin may be produced by aging, by exposure to ultraviolet light, by hormonal effects and by disease.

Problems of Sample Collection for Skin Lipid Analysis

Great difficulties are encountered in definition of a meaningful skin lipid sample, as well as in isolation of such a sample. It cannot be overemphasized that definition must precede isolation.

The lipids of the skin surface arise in part from sebaceous gland sebum, a mixture of lipids and cellular debris, and from the products of normal epidermal keratinization. Eccrine sweat glands, as well as apocrine sweat glands, may contribute to the surface lipids. Lipases and other enzymes of the surface and follicular microflora may modify the surface lipid sample. The lipids of the microflora themselves may contaminate the sample. Endogenous enzymes, such as esterases, in the sebaceous gland duct and upper hair follicle may act on the sebaceous gland lipids before they reach the skin surface.

A wide variety of lipids and other organic substances in the environment find their way onto the skin surface and appear in analytical results unless great care is taken. Cosmetics, especially those containing lanolin (sheep surface lipid) are serious offenders. Oxidative degradation of skin surface lipids may also occur. These factors may be reduced by prior removal of skin surface lipids so that only the recently formed lipids are sampled.

According to limited data available, variations in the amounts and types of lipids occur in different areas of the body. The variations reflect primarily, however, the number and size of the sebaceous glands in the areas of skin studied (9).

The metabolic activity of the inevitable bacterial contaminants of the skin constitutes a great problem of skin lipid sampling. Studies of internal lipid metabolism usually begin with a sample collected under sterile conditions.

Another problem is the collection of samples sufficient in size for lipid analysis. The development of sensitive techniques, particularly thin-layer and gas-liquid chromatography for the analysis of lipids, however, has somewhat alleviated this difficulty.

The solvent used for the extraction of lipid samples from *in vivo* sources represents a severe limitation in the analysis of skin lipids. Nonpolar solvents, such as hexane, pentane, and petroleum ether, remove relatively nonpolar lipids but fail to extract significant

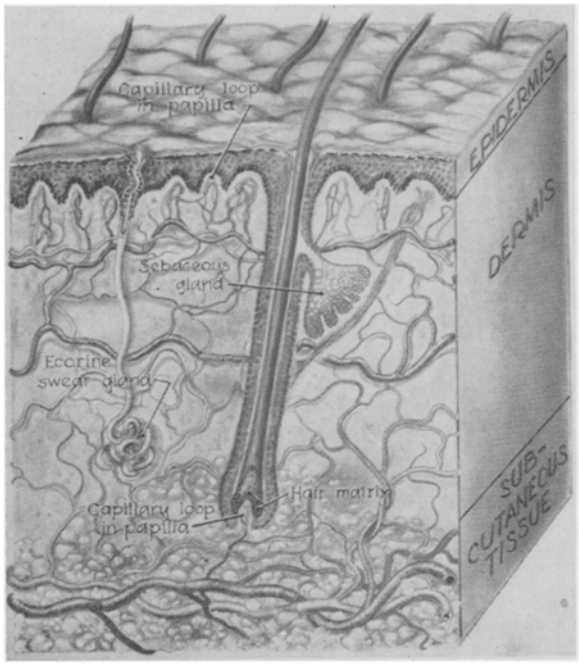

FIG. 1. Diagrammatic representation of human skin. (Reproduced by permission from Pillsbury, D. M., W. B. Shelley and A. M. Kligman, "A Manual of Cutaneous Medicine," W. B. Saunders Company, Philadelphia, 1961).

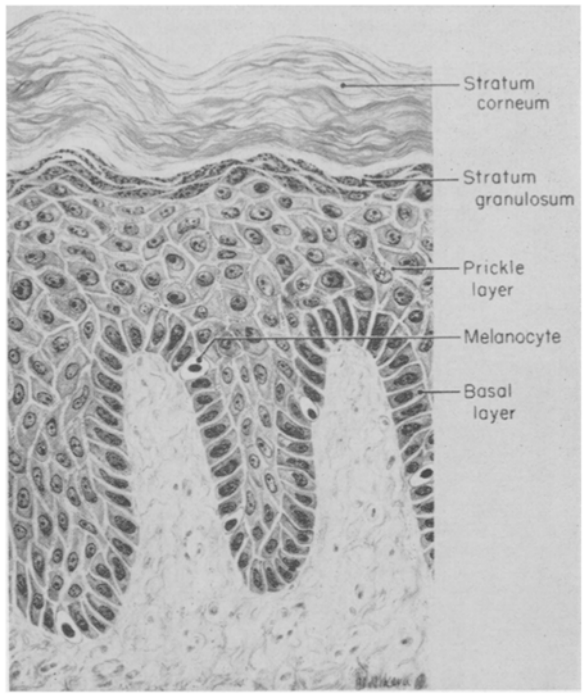

FIG. 2. Diagrammatic representation of the layers of the human epidermis (Reproduced by permission from Pillsbury, D. M., W. B. Shelley and A. M. Kligman, "A Manual of Cutaneous Medicine," W. B. Saunders Company, Philadelphia, 1961).

amounts of more polar lipids. The polar lipids will be extracted with a combination of chloroform/methanol 2/1 by volume, but this solvent is highly irritating and causes significant damage to intact skin. Lipid solvents of moderate polarity, such as diethyl ether, appear to be a suitable compromise (10,11) for surface lipid removal. The combination of chloroform/methanol 2/1 can be used, however, for *in vitro* lipid extraction.

Review of Methods for Collection of Lipids of the Skin

A. *Skin Surface Lipids (12).*

1. Extraction of the lipids from the skin surface with a lipid solvent.
   a. Cup methods (5,13,14). A hollow glass cylinder which delineates a known area of skin surface is pressed tightly or cemented to the skin. A measured amount of the selected solvent is placed in the cylinder for a standardized period of time, then removed by pipette.
   b. Swabbing of the skin with solvent soaked cotton balls (15,16). It is imperative to remove the inherent lipids from the cotton balls with repeated extraction with solvent to render the cotton balls "fat-free."
   c. Immersion of a human extremity (17), or the scalp (11) in lipid solvent.
   d. The technique of collection of lipid samples by the free flow of acetone across the skin of the upper back (18) can introduce several uncontrolled variables: the duration of solvent contact with the skin, the penetration of the solvent into the upper layers of epidermis, and the rates of solubility of the various classes of skin surface lipids. Unfortunately, the lipid yield by this method was not reported.
   e. Extraction of hair fat (19). Exogenous contamination is a serious problem.
   f. Rat and mouse skin surface lipids have been obtained by immersion of an entire animal in solvent (20,21). The animals were housed in wide mesh screen cages to reduce contamination from food and feces. Each animal was anesthetized with ether, and the feet, tail and anal region were washed with acetone. The animal was dipped into 350 ml of acetone in a glass beaker 5 times during one minute with only the ears and mouth kept above the solvent surface. The yield of surface lipid was 130 to 170 mg/day/kg of body weight (20).

      We obtained rat skin surface lipids as follows (22): The rats were shaved from the neck to the base of the tail and from flank to flank with a hair clipper previously soaked in $CHCl_3$ to remove lubricating oil. Each rat was individually caged. Scrupulously cleaned cages were used, each equipped with a wide mesh screen floor to minimize contamination from feces and urine. Each animal was fitted with a large inverted conical plastic collar which prevented completely the rat's licking himself. After the rat was accustomed to his collar, the back was wiped with hexane-soaked fat-free cotton balls. A "standardized" wiping was done by one person, i.e., the same number of strokes were used in the same direction and in the same area of the back. One person held the rat, another (wearing gloves previously soaked in hexane) wiped, while a third removed feces and urine from the worktable during the wiping.
2. Extraction of lipids adsorbed onto filter paper (23), cigarette paper (5,24), or clothing (25). This technique, when used on the forehead, yields samples of sebum with only minimal contamination with epidermal lipids. Cleansing of the skin prior to the sample collection minimizes this epi-

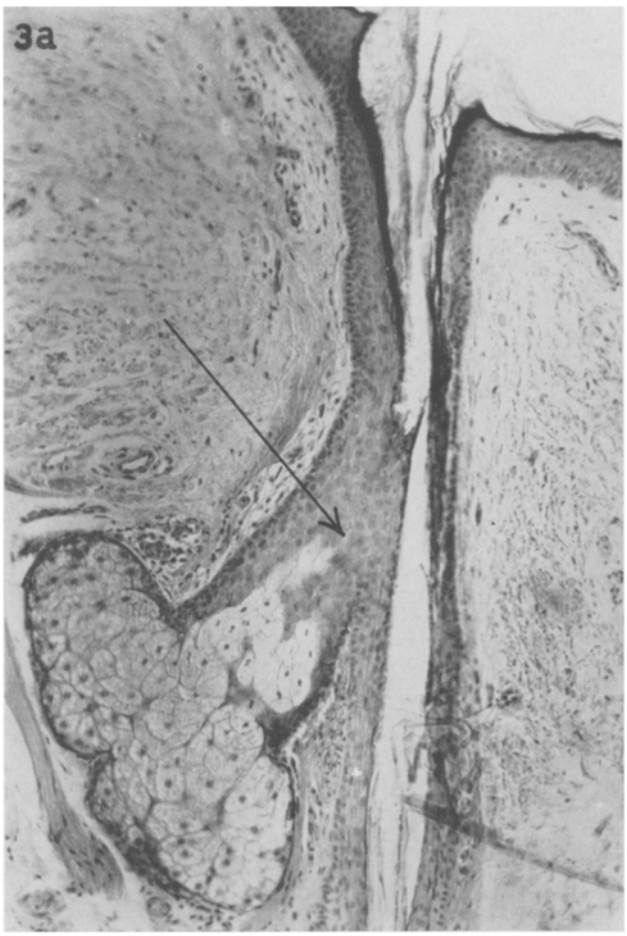

FIG. 3a. Photomicrograph of human pilosebaceous unit. Note the multilobulated sebaceous gland which opens into the follicular canal through the sebaceous gland duct (arrow). (Through the courtesy of Dr. R. R. Suskind.)

dermal contamination. This collection method has little value in the isolation of epidermal lipids, even from the sebum-free surface of the palms and soles.

3. Ablation of the sebaceous glands of the skin of the mouse with methylcholanthrene has been used to study sebaceous gland regeneration (1). This technique might be utilized to yield a skin surface free from sebaceous excretions.

B. *Ductal Lipids.* Sebaceous follicles (large sebaceous glands, with or without tiny vellus hairs, with dilated ducts which open to the skin surface) were thought to be amenable to direct cannulation. Such attempts with stainless steel capillary tubing were unsuccessful (26). This was likely due to the viscous semiliquid composition of the lipoid material in the sebaceous gland and duct.

Some investigators have reported the appearance of tiny clear droplets of sebum at the follicular openings on the skin of "good sebum producers" (5). No attempt was made, however, to collect these droplets for lipid analysis, and other workers have failed to duplicate these findings.

The material expressed from comedones ("blackheads" and "whiteheads") consists of keratin, masses of bacteria, and lipids of sebaceous and epidermal origin (27).

C. *Sebaceous Gland Lipids.* As noted above, mammals vary in their sebaceous gland structures. A few examples of mammalian origin, as well as the uropygial (preen) gland of the goose used in subsequent study (53,55) are discussed.

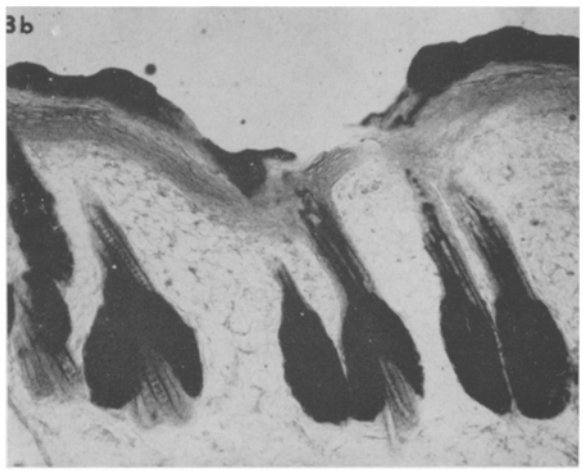

FIG. 3b. Photomicrograph of rat skin, colored with Sudan Black, ×100. Note the dark globules of lipid which fill the sebaceous glands, extend into the hair follicle canal and cover the skin surface.

1. Human sebaceous gland lipids. A technique for the isolation and collection of individual human sebaceous glands has been developed (28). The isolation and collection technique, however, is tedious and time-consuming. Scalp skin is used because of its relatively high sebaceous gland population. The yield of lipid is about 10 μg per sebaceous gland. Total samples, which range from 0.5 mg to 1.0 mg of lipid, can be obtained from a segment of scalp skin approximately 1 cm × 4 cm in size. This represents about 50–100 individual sebaceous glands.
2. Meibomian gland lipid (29). Lipids from the Meibomiam glands of the eyelid can be obtained by gentle pressure of the anesthetized eyelid against a glass rod. The semisolid, whitish material is then removed from the edge of the eyelid with a flat spatula and put directly into solvent.
3. Rat preputial gland. This modified sebaceous gland, a 2 cm long sac-like gland in the subcutaneous tissue adjacent to the urethra, has been utilized for the study of *in vitro* lipogenesis (30,31). The whole gland was extracted for lipid analysis.
4. Goose preen gland (uropygial gland). This modified sebaceous gland represents the only sebaceous organ of the domestic goose. The preen glands are sac-like structures, approximately 3 cm long, located at the base of the tail. The contents of the excised gland can be expressed.

D. *Epidermal Lipids.*

1. Total epidermis. Many techniques have been used for the separation of the epidermis from the dermis. These include physical stretching of the skin (32–34), heat (35), chemical treatment with inorganic salts, acids (such as acetic acid), and bases (such as NH_4OH) (35–37), and digestion with enzymes, mainly of pancreatic derivation (38–41). The usual hazards of contamination and alteration of lipids must be carefully examined and reported in detail. The complete separation of the two fractions is

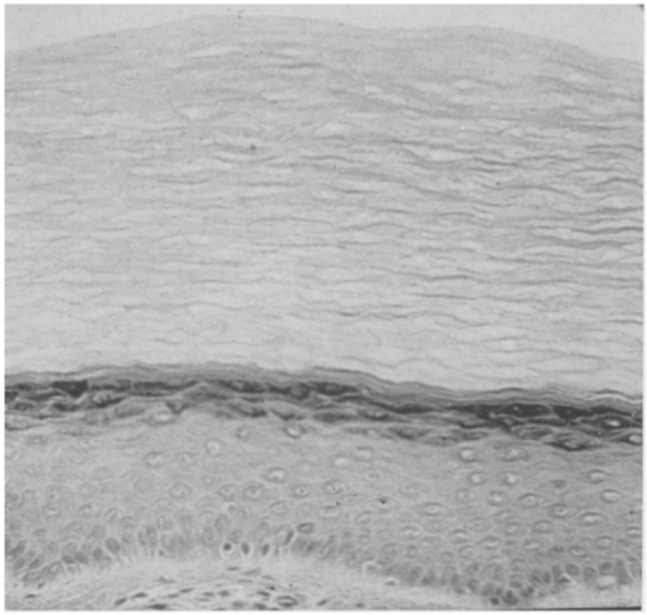

FIG. 4. Photomicrograph of normal human epidermis from the palm. Hematoxylin-eosin stain; ×105. Through the courtesy of Dr. Walter C. Lobitz, Jr.) Note the thickened horny layer (stratum corneum).

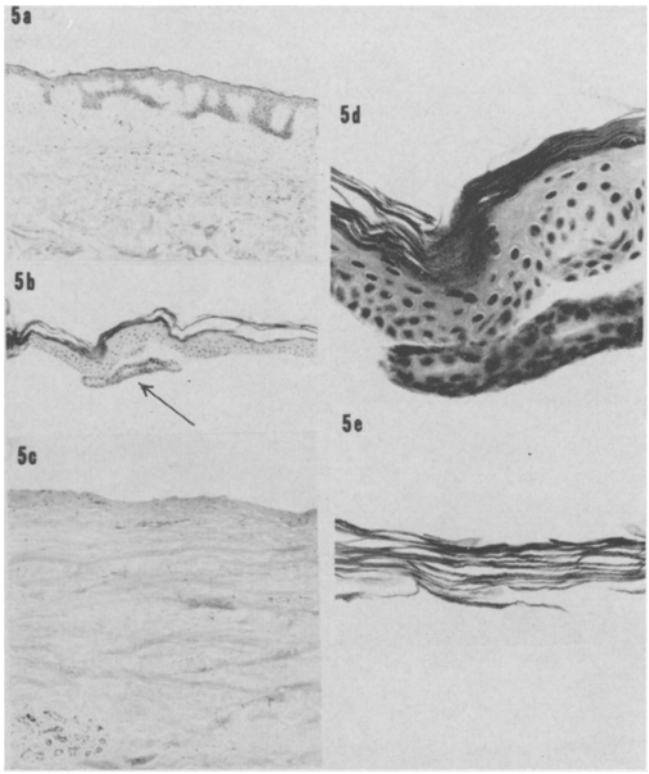

FIG. 5a. Photomicrograph of human skin.

FIG. 5b. Photomicrograph of human epidermis which has been separated from the dermis by treatment with NH_4OH. The arrow indicates the dermal segment of an eccrine sweat duct displaced from its normal position perpendicular to the skin surface and lying free under the separated epidermis.

FIG. 5c. Photomicrograph of human dermis after the epidermis has been separated by NH_4OH treatment. An eccrine sweat gland is visible at lower left.

FIG. 5d. Photomicrograph of separated human epidermis under higher magnification.

FIG. 5e. Photomicrograph of human stratum corneum from which the living epidermis has been removed by treatment with trypsin.

best controlled by histologic sections. Care must also be exercised to avoid contamination with subcutaneous fat (42).

Blank *et al.* (43) described the use of the Castroviejo keratotome to obtain thin sheets of skin. The collection of *epidermal* sheets 0.1 mm in thickness as described by Blank *et al.*, however, is difficult to reproduce in our experience. Halprin concurred in this finding but pointed out that samples of 0.2 mm in thickness can be reproduced regularly. This sample, however, contains 10–25% dermal contamination. Samples of 0.15 to 0.18 mm in thickness minimize this dermal contamination (44).

Patterson and Griesemer (45) utilized 8 mm diameter punch biopsies of skin to study *in vitro* lipogenesis (46,9). Free-hand sections were cut from the cylinder of skin parallel to the skin surface. The upper, or "epidermal," section histologically was found to contain all the epidermis and a layer of upper dermis while the lower or "dermal" section contained the remainder of the dermis with the sebaceous glands.

2. Stratum corneum ("dead epidermis"). The conventional isolation of stratum corneum with cellophane tape stripping (47,48) is unsuitable for lipid analysis since the tape yields material that gives a rapid Liebermann-Burchard test (34). Two alternative methods, dilute trypsin digestion of the living epidermis after epidermal-dermal separation with ammonia, or the separation of the stratum corneum with cantharidin, supply a stratum corneum suitable for lipid analysis (49).

   Pathologic scales of the epidermis have been utilized for various lipid studies (50,51).

3. Living epidermis. The lipids of the living epidermis can be extracted after the stratum corneum is removed from the total epidermis by dilute trypsin digestion (49).

E. *Dermal Lipids.* After complete epidermal-dermal separation is effected by methods noted above, the dermal lipids can be extracted from the dermal fraction. This fraction, however, contains sebaceous glands. Skin free of sebaceous glands from the palms or soles, should yield a valid sample of dermis.

Preparation of Skin Lipid Samples

Skin lipid samples, which fall roughly into three categories, were prepared: 1) epidermal lipid samples, 2) lipid samples from various sebaceous type glands, and 3) lipid samples from the skin surface. These samples have been analyzed by various techniques (53–55). Table I summarizes data pertaining to these tissues.

Preparation of Tissue. Samples No. 1 and No. 2 (Table I) were obtained from leg amputations. Only "normal" skin was selected, on the basis of visual appearance (Fig. 5a). The subcutaneous fat was removed; the skin was cut into pieces about 5 cm × 15 cm, frozen immediately on dry ice, and sealed in plastic bags in a nitrogen atmosphere. The skin was stored in the freezer at −20C until it was analyzed.

To obtain its area, the skin was placed dermis side down on aluminum foil and outlined with a sharp point. The aluminum foil tracing was cut out and retraced on bond paper of uniform weight. The weight

of the tracing was compared to the weight of a known area of paper and the area of the skin was computed.

To separate epidermis from dermis, the thawed skin was spread on a glass plate (epidermis up) and exposed to ammonia fumes (49). Lipid degradation by NH_4OH probably does not occur, since concentrated NH_4OH shaken with brain lipids for 10 min caused no degradation (52). A rectangular chamber for the development of thin-layer chromatographic plates was convenient for this separation. The skin was placed in the chamber above a petri dish containing about 15 ml concentrated NH_4OH and the chamber was sealed. After 1 hr of exposure, the epidermis was easily teased off the dermis (Figs. 5b, 5c, 5d). The hair usually remained with the dermis.

The epidermis was then either extracted immediately or a further separation into stratum corneum and living epidermal tissue was made (No. 4 and No. 5, respectively, of Table I). For the latter separation a piece of Whatman 3MM paper (previously extracted with chloroform/methanol 2/1 and dried) was placed in a flat porcelain tray. The paper was then moistened with a 0.1% solution of trypsin (Worthington TRL 6250) in 0.5% $NaHCO_3$. The skin was placed basal layer side down on the moistened paper on the tray covered with transparent plastic sheeting (Saran Wrap) so that a N_2 atmosphere could be provided. After 1 hr the stratum corneum was removed from the living layer cells which stuck to the filter paper. To rinse off any remaining cells the sheet of stratum corneum was then floated on a few milliliters of water. Histologic sections were then taken (Fig. 5e).

Skin tissue from four rats was obtained after they were killed by ether inhalation. The preputial glands were first removed carefully to avoid inclusion of surrounding tissue. The glands were individually weighed and pooled for extraction. The hair was clipped from the skin of each rat, weighed and pooled for extraction. Before use the clipper blades were cleansed with chloroform/methanol 2/1 to avoid contamination with lubricating oil. The skin from most of the body was then removed and weighed. Its area was traced and the epidermis separated from dermis as described above. With rat skin, the hair follicles remained with the epidermis.

Individual human sebaceous glands were harvested by the method described in section C. 1. The harvested glands were put directly into chloroform/methanol 2/1.

Lipoid material was expressed from the Meibomian gland and dissolved in chloroform/methanol 2/1.

Extraction of Lipids. All epidermal samples (Nos. 1 through 6) were individually extracted with chloroform/methanol 2/1. (Only redistilled solvents were used.) The extraction was performed in a Virtis or a Kontes homogenizer with a volume of solvent (in ml) equal to 10 times the weight (in gm) of tissue. After each homogenization the slurry was filtered through a sintered glass funnel (medium porosity) and the residual tissue reextracted. This extraction was repeated until negligible lipid remained (3 to 4 times). To extract the lipids from living epidermal cells stuck to the filter paper, the paper and cells were homogenized in a heavy walled flask with a thick glass rod in a solvent volume 10 times the tissue weight (in grams).

The results of the analysis of these samples are reported in the succeeding papers (53–55).

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