Localization of sites of lipid biosynthesis in mammalian epidermis

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Abstract The end-product of epidermal differentiation is a stratified layer of corneocytes whose extracellular lipid bilayers provide a permeability barrier. It is generally accepted that the epidermis synthesizes most if not all of the lipids found in this tissue and that extra-epidermal tissues contribute very little to this lipid content. Moreover, the individual epidermal strata in which epidermal lipid biosynthesis occurs are not known. To address this question, we examined [^{3}H]H_2O incorporation into nonsaponifiable and saponifiable lipids in individual epidermal cell layers 3 hr after intraperitoneal injection into neonatal mice, and compared this to protein and DNA synthesis using intraperitoneal [^{3}H]leucine and [^{3}H]thymidine incorporation, respectively. Lipid biosynthesis was also assessed by [^{14}C]acetate incorporation into lipid fractions in organ cultured skin and in epidermal subpopulations. The in vivo studies demonstrated that the biosynthetic activity of both saponifiable and nonsaponifiable lipids was comparable to, if not greater, in the stratum granulosum (SG) than in basal/spinous (SB + SS) layer, despite significantly lower levels of both protein and DNA synthesis in the SG. On a mass basis, the SG accounts for about four times the biosynthetic activity of the combined SB + SS layers. The lipid biosynthetic activity in vitro also was two- to fivefold higher in the SG, regardless of whether the epidermis was separated into individual cell layers before or after incubations with radiolabel. Moreover, this difference could not be ascribed to increased acetate pools or to elevated metabolism in the SG versus the SB + SS since the rates of CO_2 production were much lower in the SG fraction. The increase in lipid biosynthesis in SG over SB + SS was greatest for phospholipids, followed by glycosphingolipids, and free sterols but was observed in almost all lipid classes. These studies show not only that mammalian epidermis is an active site of de novo lipid biosynthesis, but also that this activity remains high in the stratum granulosum, while other forms of metabolic activity are diminishing. These observations are consistent with the knowledge that lipids extruded from the stratum granulosum layer provide the hydrophobic permeability barrier, and further suggest that elevated synthetic activity in the stratum granulosum would allow rapid replenishment in the event that the barrier is damaged.—**Monger, D. J., M. L. Williams, K. R. Feingold, B. E. Brown, and P. M. Elias.** Localization of sites of lipid biosynthesis in mammalian epidermis. *J. Lipid Res.* 1988. **29:** 603–612.

Supplementary key words stratum granulosum • stratum corneum • stratum spinosum • stratum basale

The stratum corneum of terrestrial mammals forms a two-component system of lipid-depleted cells embedded in an intercellular matrix enriched in hydrophobic neutral lipids and ceramides (reviewed in references 1 and 2). Several independent lines of evidence suggest that these lipids are critical for epidermal waterproofing (3–6). Although the epidermis is capable of synthesizing a broad spectrum of lipids (7–9), the layers where lipid biosynthesis occurs are not known. Moreover, the relative roles of extracutaneous versus cutaneous sources of those lipids important for cutaneous waterproofing are unknown. Recent studies suggest, however, that the cutaneous sterologenesis is virtually autonomous regarding the influence and contributions of circulating lipoproteins (10). Both postconfluent cultured keratinocytes (11) and mature hair follicles (12) lack LDL receptors. Moreover, the skin is a major site of total body sterol synthesis, with the epidermis accounting for about 30% of cutaneous sterologenesis (13). Very recent studies have shown that the epidermal basal cell layer does elaborate LDL receptors (14), and that preconfluent keratinocytes both display LDL receptors (15) and regulate sterol synthesis in response to exogenous lipoproteins (15, 16). Thus, a picture is emerging that the proliferating compartment of the epidermis may be regulated to an unknown extent by extracutaneous influences, while sterologenesis in the postmitotic, differentiating layers would not be affected substantially. In contrast, the epidermis may be more dependent on exogenous fatty acids, since it lacks the Δ^6 desaturase system needed to generate eicosanoids from essential fatty acids (17). Yet, again, recent studies have demonstrated high levels of epidermal fatty acid synthesis, independent of circulating levels (9). Moreover, despite

Abbreviations: LDL, low density lipoproteins: SG, stratum granulosum; SC, stratum corneum; SB, stratum basale; SS, stratum spinosum; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

some degree of autonomy from the circulation, sterologenesis and fatty acid synthesis are regulated by cutaneous barrier requirements (6, 9, 18).

In this study, we have quantitated the rates of lipid biosynthetic activity in separate epidermal cell layers. Surprisingly, the highest rates of de novo synthesis, both in vivo and in various in vitro systems, occurred in differentiating, outer epidermal cell layers, rather than in the proliferating compartment. Thus, a large proportion of epidermal lipid biosynthesis may be directed towards provision of the cutaneous permeability barrier, rather than in supplying lipids for membrane synthesis in response to requirements for cellular growth and replication.

METHODS

Materials

ICR neonatal mice (12–48 hr) were purchased from Simonsen Laboratories (Gilroy, CA). [2-^{14}C]Acetic acid, sodium salt (50 mCi/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Tritiated water (1 Ci/g) was purchased from New England Nuclear Research Products/DuPont (Boston, MA). [Methyl-^{3}H]thymidine (88 Ci/mmol) and L-[4,5-^{3}H]leucine (120 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Staphylococcal exfoliative toxin was purchased from Toxin Technology (Madison, WI). Silica gel 60 thin-layer chromatographic (TLC) plates (20 × 20 cm) and high performance TLC plates (20 × 20 cm) were purchased from E. Merck (Darmstadt, West Germany). DME-H21 media and fetal calf serum (FCS) were purchased from Whittaker M.A. Bioproducts (Walkersville, MD) and supplemented, as described previously (13). All solvents used were of reagent grade, purchased from Allied Fisher Scientific (Springfield, NJ).

Lipid biosynthesis in epidermis in vivo

Groups of neonatal mice (four or five groups of 4–11 mice each) were injected intraperitoneally with 5 mCi (50 μl) of [^{3}H]H_2O. After 1 hr, animals were injected subcutaneously with a purified fraction of staphylococcal exfoliative toxin (30 μg/100 μl), and the animals were placed in an incubator at 34°C. At the end of 3 hr, animals were killed by cervical dislocation, and the stratum granulosum + stratum corneum (SG + SC) layers were peeled off as intact sheets while the stratum basale + stratum spinosum (SS + SB) cells were scraped from the denuded surface with a #10 surgical blade. This process has been shown previously to remove cells from above the basement membrane without dislodging dermal elements (6, 13, 19). Previous studies have shown that the staphylococcal epidermolytic toxin causes cell separation at the SG/SS interface with no morphological evidence of cytotoxicity (cited in ref. 19). All cells were placed immediately in ice-cold phosphate-buffered saline (PBS) and kept frozen in liquid nitrogen until all samples were collected. Samples were thawed and SG + SC sheets were incubated in 0.5% trypsin for 2 hr at 37°C. This procedure was shown not to contribute significantly to the protein content present in the two cellular preparations. The SG cells were vortexed off the residual SC sheets, filtered through cheese-cloth, pooled with subsequent washes, pelleted, and washed in PBS. To insure uniform incubation time periods, SB + SS cells were treated similarly, but without trypsinization. Tissues were saponified in 45% ethanolic potassium hydroxide, acidified, extracted three times with petroleum ether, chromatographed on TLC plates, and the radioactivity was determined by liquid scintillation spectrometry using a Beckman Model LS-1800 counter. The one-dimensional TLC solvent system consisted of: *a)* diethyl ether developed 2 cm from origin, followed by *b)* petroleum ether–diethyl ether–acetic acid 80:20:1 (v/v/v) developed 14 cm from origin, followed by *c)* petroleum ether developed to the top (19).

DNA and protein synthesis in vivo

Neonatal mice (n = 3 to 7) were each injected intraperitoneally (IP) with 50 μCi (50 μl) of [^{3}H]thymidine and incubated in a moist atmosphere at 37°C for 4 hr. Two parallel groups of mice were injected IP with either 50 μCi of [^{3}H]leucine per animal or with 5 mCi of [^{3}H]H_2O per animal and incubated under identical conditions. One hour after the start of each incubation, animals were injected subcutaneously with aliquots of the staphylococcal exfoliative toxin and incubated and killed as described above. SB + SS and SG cellular preparations were obtained, as described above. For total mass determinations, aliquots of each layer were desiccated for 24 hr over phosphorous pentoxide. Parallel samples were precipitated with 10% cold trichloroacetic acid (TCA); the precipitates were washed repeatedly with cold TCA, followed with ethanol, and resuspended in TCA at 90°C for 15 min. Following centrifugation, the supernatant was separated from the pellet, the protein pellet was redissolved in 1% sodium dodecyl sulfate (SDS), and aliquots were taken for liquid scintillation spectrometry and for measurement of protein content (see below).

Lipid biosynthesis in organ cultured epidermis

To assess synthesis in organ cultured skin, two methods were used. In the first method, subcutaneous fat was scraped off full-thickness neonatal mouse skin and ~1-cm^2 skin pieces were floated on DME-H21 with 5% FCS plus antibiotics in 100-mm Petri dishes at 37°C. The organ cultures were labeled with [^{14}C]acetate (50 mCi/mmol, 20 μCi/ml) for 3 hr. Dithiothreitol (DTT, 10 mM final concentration), which causes an intraepidermal split above the basal and spinous layers and beneath the granular layers (6, 13), was added to the incubation solution for the last

30 min. The medium was decanted, saved, and frozen, and the cultures then were transferred onto iced PBS. An intact sheet of SG + SC then was peeled off, and the SB + SS cells were scraped off the denuded surface, as described above. Both samples were immediately freeze-thawed three times. The SG + SC sheets were either incubated with trypsin, as described above or, in cases where protein is used as a denominator, the SG was sheared off the SC sheets using a loose-fitting glass homogenizer, and subsequently filtered through cheese-cloth. SB + SS and SG samples were sonicated with a probe (Fisher Model 300 Sonic Dismembrator) using five pulses of 15 sec each. Aliquots were taken from each sample for measurement of DNA content and the remainder was extracted for lipids using the method of Bligh and Dyer (20) (see below). The media samples also were solvent-extracted to monitor the extent of loss of incorporated lipid into the medium.

In the second method, full-thickness neonatal mouse skin, prepared as described above, was incubated with [^{14}C]acetate (50 mCi/mmol, 20 μCi/ml) at 37°C for 4 hr. Exfoliative toxin (500 μg/ml final concentration) was added during the last 1.5 hr and the samples were washed three times with ice-cold PBS. The SG + SC sheet was peeled off and the SB + SS cells were scraped off the dermis. SG cells were obtained either by trypsinization or by shearing, as described above. After washing, SG and SB + SS cells were freeze-thawed and sonicated, aliquots were removed for either protein or DNA determinations (see below), and the lipids were either saponified in 45% ethanolic KOH, acidified and extracted with petroleum ether, or extracted by the method of Bligh and Dyer (20). Lipids were fractionated and biosynthetic activity was quantitated as described below.

Lipid biosynthesis in individual epidermal cell layer preparations

To assess lipid biosynthesis in previously separated, individual cell layers, neonatal mice were injected with exfoliative toxin as described above, and incubated in a moist atmosphere at 37°C for 2 hr. Animals were killed by cervical dislocation, and the SG + SC sheets were peeled off the carcasses, while the SB + SS cells were scraped off the dermis, as described above. SG cells were sheared off the SC using a loose-fitting glass homogenizer, and each cell layer then was incubated with [^{14}C]acetate (20 μCi/ml) at 37°C for 3 hr in DME-21 with 5% FCS plus antibiotics. The cell preparations were washed in PBS, sonicated, and aliquots were obtained for lipid extraction, fractionation, and scintillation spectrometry, as well as for DNA and protein determinations (see below).

Lipid extraction, fractionation, and quantitation

Lipids were extracted by the method of Bligh and Dyer (20), modified as previously reported (21). Radiolabeled samples were cochromatographed with authentic standards on silica gel 60 thin-layer chromatographic plates using the following solvent systems (21). Glycosphingolipids and ceramides were subfractionated in chloroform–methanol–water 90:10:1 (by vol) to the top of the TLC plate, dried and rechromatographed in petroleum ether–diethyl ether–acetic acid 70:50:1 (by vol). The neutral lipids above the ceramides and the polar lipids below the glycosphingolipids then were reextracted and subfractionated. Neutral lipids were chromatographed in petroleum ether–diethyl ether–acetic acid 80:20:1 (by vol) developed 12 cm up from the origin, dried, and rechromatographed to the top of the TLC plate in 100% petroleum ether. Polar lipids and cholesteryl sulfate were chromatographed in tetrahydrofuran–methylal–methanol–4 M ammonia 60:30:10:4 (by vol) to the top of the TLC plate, dried, and rechromatographed in chloroform–methanol–acetic acid–water 60:35:0.5:4.5 (by vol) to 14 cm from the origin. All individual lipid bands were visualized under a UV-A light source after spraying with 0.2% aqueous 8-anilino-sulfonic acid, re-extracted off the silica gel with Bligh-Dyer solution, and aliquots were taken for weight (Cahn Balance) and/or liquid scintillation spectrometry.

Other biochemical assays

Protein and DNA assays. Proteins were assayed using bicinchoninic acid and cuprous ion reagent (22), as outlined in an enhanced microassay protocol (Pierce Chemical Co., Rockford, IL). Samples were measured in triplicate, using bovine serum albumin as the standard. DNA was assayed using bisbenzimidazole, as described by Labarca and Poigen (23); calf thymus DNA was used as a standard.

CO_2 production. Aliquots from the same SB + SS and SG cellular preparations that were utilized for the lipid biosynthetic studies were suspended in DME-H21 plus 5% FCS for measurement of CO_2 production from acetate. Cells were incubated with [^{14}C]acetate (10 μCi) at 37°C at final acetate concentrations of 1×10^{-2} M, 1×10^{-3} M, 2×10^{-4} M, or 1×10^{-4} M for 3 hr in sealed flasks containing 0.5 N NaOH in a separated, central well. Incubations were stopped by the addition of 2 N H_2SO_4 to the cell suspension, warmed at 37°C for an additional hour, and stored sealed overnight at 4°C. Radioactivity was measured as [^{14}C]Na_2CO_3 collected in the central well, correcting for quenching of radioactivity by the NaOH.

Statistical significance

Statistical significance for all data was determined using a two-tailed Student's t test.

RESULTS

Localization of epidermal lipid biosynthesis in the whole animal

To approach the issue of lipid biosynthetic sites in the epidermis, we used both whole animal and in vitro systems.

TABLE 1. Sites of lipid biosynthesis in neonatal mouse epidermis

Experiment[a] (No. of groups; animals/group)	Biosynthetic Activity[b]		
	Basal + Spinous	Granular	SG/SB + SS Ratio
Exp. 1 (4; 4)	37.1 ± 7.2	39.9 ± 7.5	1.1
Exp. 2 (4; 4)	40.9 ± 10.2	85.8 ± 21.6	2.1
Exp. 3 (5; 11)	43.3 ± 6.6	54.0 ± 5.8	1.2

Neonatal mice were incubated for 3 hr with $[^3H]H_2O$ (5 mCi each) and exfoliative toxin (30 μg each) in vivo and then killed. Epidermal cellular populations were isolated, saponified, and extracted for lipid. Lipid biosynthetic rates are corrected for the specific activity of the $[^3H]H_2O$ measured in the pooled serum from each group of animals.

[a] $[^3H]H_2O$ in serum (mCi/mol): Exp. 1, 36.9 ± 1.6; Exp. 2, 59.2 ± 5.5; Exp. 3, 37.6 ± 2.2.

[b] Values are given as nmol of $[^3H]H_2O$ incorporated into lipid/mg protein · hr^{-1}; mean ± SEM.

In the first approach, neonatal mice were labeled with intraperitoneally (IP) administered $[^3H]H_2O$ over a 3-hr period, and the incorporation into specific lipid fractions within various epidermal cell layers was compared. As seen in **Table 1,** three independent experiments were performed to measure overall lipid biosynthetic activity. The ratios of lipid biosynthesis in the SG versus the SB + SS preparations ranged from 1.1 to 2.1. Moreover, these data show that sustained rates of lipid biosynthesis, measured in these layers, reflected both saponifiable and nonsaponifiable lipid biosynthesis, since the increase in total sterol synthesis and total fatty acid synthesis (increased 1.2- and 1.4-fold, respectively, in SG vs. SB + SS) were comparable (**Table 2**). The experiment-to-experiment variations in the ratio of biosynthetic activity may reflect variable toxicity resulting from slight differences in the length of exposure and/or total volume of staphylococcal epidermolytic toxin administered to animals (individual animals were not weighed and each received a 0.1-ml aliquot; see Methods).

To determine whether the high level of lipid biosynthetic activity observed in the granular layer was a nonspecific phenomenon attributable to an increase in the overall metabolic activity of this layer, we compared lipid (from ^3H_2O), protein (from [^{3}H]leucine), and DNA (from [^{3}H]thymidine) synthetic rates in tissues prepared under identical conditions from parallel groups of animals. As seen in **Table 3,** the rates of both protein and DNA synthesis were significantly lower in the SG versus the SB + SS layer preparations (SG/SB + SS ratios of 0.30 and 0.033, respectively). Thus, it is clear that lipogenesis is specifically elevated, in comparison to other types of synthetic activity, in the granular layer, and increased lipid biosynthesis in the granular layer cannot be attributed to either nonspecific activation of the SG layer in comparison to the SB + SS layers, nor to selective toxicity towards one preparation versus others from the reagents used to isolate the various tissues.

To determine the percentage contribution of the SG versus the SB + SS layer fractions to the overall rates of epidermal lipogenesis, we quantitated the dry mass contributed by each layer (see Methods). As can be seen in **Table 4,** the granular layer contains threefold greater mass than the SB + SS layer (protein is 80–90% of the dry mass for both cell fractions; data not shown). Hence, the contribution to overall epidermal lipogenesis by the SG is approximately four times that of the combined SB + SS layers. It should be noted that the major portion of epidermal mass is present in the stratum corneum which consists of lipids and anucleate cornified cells.

Localization of lipid biosynthesis in organ cultured whole skin

In vivo studies, such as the ones described above, leave open the possibility of potential contributions from extracutaneously synthesized lipids that have been transported to the epidermis. Hence, we next studied lipid biosynthesis in organ cultures of whole skin incubated with [^{14}C]acetate for 3 to 4 hr followed immediately by further separation of the epidermis into individual cell layers. In these studies, the overall amount of [^{14}C]acetate incorporated into lipids was small: 1×10^4 dpm/cell layer/animal in SB + SS and $3.9–4.4 \times 10^4$ dpm/cell layer/animal in SG (0.002 and 0.01% incorporated, respectively). As seen in **Table 5,** the rates of lipid biosynthesis, normalized to protein content, were higher in the SG than in the SB + SS

TABLE 2. Lipid biosynthetic activity in neonatal mouse epidermal cell layers

Fraction	Biosynthetic Activity[a]		
	SB + SS	SG	SG/SB + SS Ratio
Total lipids	40.4 ± 1.8	59.9 ± 13.6	1.5
Total sterols	6.24 ± 0.75	7.65 ± 2.03	1.2
Total fatty acids	27.9 ± 1.7	39.4 ± 10.9	1.4

Lipid extractable material, after saponification, was separated by TLC. Values are averaged from data obtained from three separate experiments (see Table 1).

[a] Values are given as nmol of $[^3H]H_2O$ incorporated into lipid/mg protein · hr^{-1}; mean ± SEM.

TABLE 3. Comparison of lipid, protein, and DNA biosynthetic rates in neonatal mouse epidermal cell layers in vivo

Fraction	Specific Activity[a] SB + SS	SG	SG/SB + SS Ratio[b]	Significance
Total lipids	7,400 ± 1,630	5,520 ± 1,590	0.75	$P > 0.1$[c]
Total protein	52,600 ± 14,000	15,900 ± 2,180	0.30	$P < 0.02$
Total DNA	38,800 ± 3,630	1,270 ± 523	0.033	$P < 0.001$

Neonatal mice were incubated in vivo for 4 hr with either [^{3}H]H_2O, [^{3}H]leucine, or [^{3}H]thymidine, followed with exfoliative toxin. The mice were killed and tissues were either saponified and extracted for lipids or precipitated with TCA, washed repeatedly, and measured for radioactivity incorporated into both the DNA and protein fractions, as well as for protein content.

[a]Values are given as dpm/mg protein per hr; mean ± SD; n = 3.

[b]In a separate experiment performed in two groups of seven animals each, SG/SB + SS ratios of 0.23 and 0.024 were obtained for protein and DNA synthesis, respectively.

[c]Not significant.

fraction ($P < 0.05$). These differences became even more pronounced when the lipid biosynthetic activity was normalized to DNA ($P < 0.01$), but lower DNA content may reflect the extensive degradation of DNA known to occur in the granular layer.

The biosynthetic activity of the major lipid species (per μg of protein) synthesized in each cell layer is shown in **Table 6.** The incorporation of acetate into most of the neutral lipid and sphingolipids and all of the phospholipid species is greater in the SG than in the SB + SS layers (Table 6). The relatively high rates of monoacylglycerol synthesis in the SG could, in part, reflect triacylglycerol breakdown during incubations, extractions, and/or fractionations. Specifically, glycosphingolipids, free sterols, ceramides I, cholesteryl sulfate, and all of the identified phospholipid species exhibited a significantly greater increase in the rate of synthesis in the SG in comparison to the same species in the SB + SS layers (two- to fivefold). In contrast, the rates of synthesis of certain nonpolar lipids (triacylglycerols, free fatty acids, and steryl esters) were not increased in the SG (Table 6).

Since granular layer lipids are secreted into the extracellular environment to form the intercellular matrix of the stratum corneum, the levels of incorporation in SG might be spuriously low if substantial amounts of lipid are released into the medium. Therefore, we compared the labeled lipids in the medium versus tissue during incubations. In the various experiments, the percentage of total incorporated cellular counts found in the medium had ranges of 0.5–1.2% glycosphingolipids, 3.3–12.2% ceramides, 0.2% free sterols, and 0.5–1.0% free fatty acids. Hence, the amount of loss was relatively small, and cellular extracts can be assumed to be representative of tissue biosynthetic activity in each fraction.

Lipid biosynthesis in separate epidermal cell populations

Although the studies in organ-cultured whole skin point to sustained high rates of lipid biosynthesis in the SG, it could be argued that lipids synthesized in the dermis or the SB + SS might translocate to the SG layer during incubations. Therefore, we next studied lipid biosynthetic rates in isolated epidermal strata (see Methods). These experiments again demonstrated elevated rates of synthesis of most lipid species (SB/SB + SS ratio of 7.5 overall) in the SG versus the SB + SS layers (**Fig. 1**).

TABLE 4. Relative contributions of each epidermal fraction to total epidermal lipogenesis

Fraction	Distribution Dry Weight	Distribution % of Epidermis[b]	Incorporation[a] Total Lipid	Incorporation[a] Total Sterols	Incorporation[a] Total Fatty Acids
	mg		*nmol [^{3}H]H_2O per layer per animal/hr*		
SB + SS	2.20	8.05	88.9	13.7	61.4
SG	6.46	23.7	287.0	49.4	254.5
SG/SB + SS ratio	2.9		4.4	3.6	4.1

[a]Incorporation data are from experiments 1–3 (cf, Tables 1 and 2), here normalized to dry weight per layer per animal per hr.

[b]The residual 68% of the epidermis represents the stratum corneum, which consists of multiple layers of anucleate cornified cells.

TABLE 5. Lipid biosynthesis in epidermal cell layers isolated from organ-cultured whole neonatal mouse skin

Biosynthetic Activity	Layer (Mean ± SEM) SB + SS	SG	Ratio SG/SB + SS
dpm/μg DNA · hr^{-1}	14.0 ± 2.0	69.5 ± 6.4**	5.0
dpm/μg protein · hr^{-1}	1.16 ± 0.75	2.41 ± 0.9*	2.1

Cleaned full-thickness neonatal mouse skin sections were incubated with [^{14}C]acetate (200 μCi/4 μmol per plate), followed with DTT, and separated into the basal + spinous layers and stratum granular + corneum sheets. SG and SB + SS cellular preparations were washed, sonicated, and aliquots were taken for measurement of DNA and protein content, as well as for radioactivity incorporated into lipid after extraction using the procedure of Bligh and Dyer (20).
*$P < 0.05$; **$P < 0.01$.

Since the rates of phospholipid synthesis were very high in the SG, despite a substantial decrease in polar lipid content (cf, refs. 19 and 24 vs. data in Table 6), and since sphingolipid content increases dramatically in the SG (19, 24), we considered the possibility that accelerated sphingomyelin synthesis and degradation could account for the large quantities of sphingolipids formed in the outer epidermis. However, a comparison of the synthetic rates of individual phospholipid species both in the organ cultures of whole skin (Table 6) and in previously separated cell fractions (**Table 7**) revealed a similar distribution of activity in both the SG and SB + SS layers. Specifically, sphingomyelin synthesis accounted for less than 10% of the total phospholipid synthesis both in the SG and in the SB + SS combined layers, and was not accelerated in comparison to other phospholipids in either preparation. Therefore, it is unlikely that sphingomyelin turnover alone could account for the large quantities of sphingolipids that are synthesized in the SG.

Finally, since [^{14}C]acetate was the label employed in all of the organ culture experiments, it could be argued that the differences in synthetic rates in the various epidermal layers could be ascribed to preferential shunting of acetate into alternate metabolic pools in one cell layer more than

TABLE 6. [^{14}C]Acetate incorporation into lipids of epidermal cell layers isolated from organ-cultured whole neonatal mouse skin

Fraction[a]	Layer SB + SS	SG	SG/SB + SS Ratio
	dpm/mg protein; mean ± SEM		
Neutral lipids			
Hydrocarbons[b]	79.0 ± 21.5	29.3 ± 2.3	0.4
Steryl esters/wax esters	456.9 ± 144.9	137.3 ± 13.6	0.3
Unknown #1[c]	49.9 ± 12.5	38.8 ± 5.1	0.8
Triacylglycerols	336.3 ± 89.1	328.2 ± 17.6	1.0
Free fatty acids	209.9 ± 52.5	179.3 ± 15.1	0.9
Unknown #2[d]	162.8 ± 43.6	242.0 ± 20.2	1.5
Free sterols	136.0 ± 34.1	367.6 ± 18.2***	2.7
Monoacylglycerols	75.4 ± 12.9	180.8 ± 16.1***	2.4
Origin	68.0 ± 19.7	179.0 ± 11.1***	2.6
Sphingolipids			
Ceramides I	419.7 ± 106.4	956.8 ± 178.3*	2.3
Ceramides II	240.1 ± 80.8	243.4 ± 12.8	1.0
Ceramides III	193.2 ± 74.1	142.6 ± 27.2	0.7
Acyl-glycolipids	98.8 ± 24.2	279.9 ± 47.0**	3.0
Glycosphingolipids	259.2 ± 83.4	1209.9 ± 170.5****	4.7
Phospholipids			
Cholesteryl sulfate	17.0 ± 3.6	37.3 ± 11.7	2.2
Unknown #3	37.7 ± 0.6	226.1 ± 123.0	6.0
Phosphatidylethanolamine	53.7 ± 5.4	183.3 ± 35.8**	3.4
Unknown #4	12.7 ± 1.7	13.6 ± 3.0	1.1
Phosphatidylserine + phosphatidylinositol	66.0 ± 15.2	259.8 ± 90.4	3.9
Phosphatidylcholine	301.0 ± 80.8	1595.6 ± 21.7***	5.3
Sphingomyelin	52.4 ± 12.6	223.1 ± 36.6***	4.2
Lysolecithin	14.0 ± 2.8	59.5 ± 12.2***	4.2
Origin	27.9 ± 6.4	105.1 ± 44.3	3.8

[a]Three groups; total of 82 mice.
[b]Hydrocarbons include squalene, alkanes, and nonoxygenated steroids.
[c]Unknown #1 possesses the chromatographic mobility of dialkyl-monoacyl glycerols.
[d]Unknown #2 has the chromatographic mobility of C-30 sterols, long chain alcohols, and diacylglycerols.
Significant differences: * < 0.05; ** < 0.02; *** < 0.01; **** < 0.001.

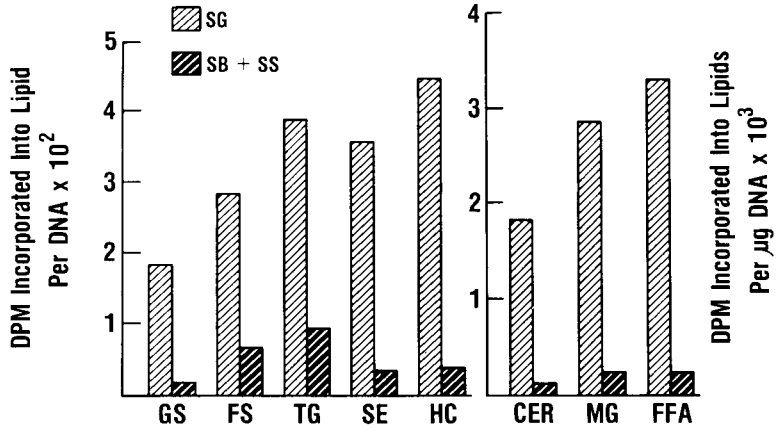

Fig. 1. Neonatal mice were injected with exfoliative toxin and killed. Basal + spinous and granular cellular preparations were obtained and incubated with [^{14}C]acetate (20 μCi/ml) for 3 hr. Tissues were sonicated, measured for DNA content, and the remainder was extracted for lipids by the method of Bligh and Dyer (20). Lipid extracts were subjected to TLC analysis along with authentic standards. Lipids were removed from the silica and measured for radioactivity.

in another. To assess this possibility, and as a further test of the viability of each preparation, we compared acetate conversion to CO_2 over a 1000-fold range of unlabeled acetate concentrations. As seen in **Fig. 2,** CO_2 generation from acetate was much higher in the SB + SS layer than in the SG layer, as expected. Thus, the high rates of acetate incorporation into lipids in the SG occur in the face of very little utilization of this substrate as an energy source. Moreover, the high rates of acetate to CO_2 conversion in the SB + SS layers, coupled with the data on thymidine incorporation into DNA and leucine incorporation into protein (cf, Table 2), provide further evidence that the differences in rates of lipid biosynthesis in various epidermal cell layers cannot be attributed to selective toxicity to the SB + SS fraction.

DISCUSSION

The most striking observation emerging from this study was that lipid biosynthesis in the stratum granulosum was comparable to, if not greater than, that occurring in the proliferative basal and spinous layers. Lipogenesis in the granular layer, although not significantly elevated over that in the basal and spinous layer, was increased markedly relative to protein and DNA synthesis. Since the granular layer possesses about three times the dry weight of the combined basal and granular layer (Table 4), the former layer demonstrates approximately four times the lipid biosynthetic activity of the latter. These results are consistent with calculations for lipid biosynthetic rates in individual cell layers of pig epidermis, where granular layer synthesis was calculated to be sevenfold greater than that in the basal + spinous layer (35.3 ± 5.8 vs. 4.9 ± 0.9 fmol/cell · hr^{-1}) (18). In marked contrast to these results, most other types of cellular biosynthetic activity shut down in the stratum granulosum, a layer that forms the transition to the anucleate stratum corneum. One exception to this is the synthesis of keratohyalin proteins, which form the interfilamentous matrix of the stratum corneum (24); synthesis of this protein(s) also peaks in the stratum granulosum (25). However, whereas the intracellular proteins of the stratum corneum serve mechanical and/or osmotic functions, granular layer lipids are delivered to intercellular domains where they mediate the permeability barrier as well as the cohesive functions of the stratum corneum (1, 2).

TABLE 7. Phospholipid synthesis in isolated neonatal mouse epidermal cell layers

Fraction	Phospholipid Synthesis[a] SB + SS	SG
Unknown #3	12.3	6.2
Phosphatidylethanolamine	15.6	15.4
Phosphatidylserine + phosphatidylinositol	19.5	24.7
Phosphatidylcholine	41.6	38.3
Sphingomyelin	6.5	8.6
Lysolecithin	2.6	4.9
Origin	1.9	1.9
	100.0%	100.0%

Neonatal mice were incubated with exfoliative toxin and killed. Basal + spinous and granular cellular preparations were obtained and incubated with [^{14}C]acetate (20 μCi/ml) for 3 hr. Tissues were extracted by the method of Bligh and Dyer (20) and these lipid extracts were subjected to TLC chromatography along with authentic phospholipid standards.

[a]Percent of [^{14}C]acetate incorporated into phospholipids.

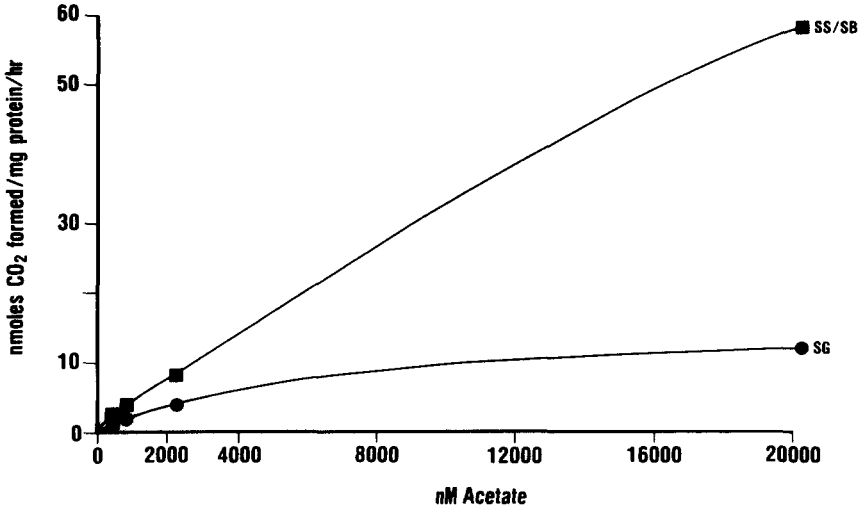

Fig. 2. SG and SB + SS cellular preparations were incubated with [^{14}C]acetate for 3 hr at 1×10^{-4} to 1×10^{-2} M concentrations in sealed vessels. [^{14}C]CO_2 was collected on 0.5 N NaOH as [^{14}C]Na_2CO_3. Recovered radioactivity is expressed as nmoles of [^{14}C]CO_2 formed per mg of protein · hr^{-1} versus nmoles of acetate present in the incubation solution.

During cornification of mammalian epidermis, a substantial shift in composition occurs from a lipid mixture consisting of ~60% phospholipids, typical of other tissues, to one lacking phospholipids, but enriched in ceramides, sterols, and free fatty acids (reviewed in refs. 2 and 8). In light of these earlier studies, it is interesting that the sphingolipid and free sterol fractions exhibited the greatest increases in synthetic rates in the granular versus the basal + spinous layers. However, virtually every lipid fraction appeared to be synthesized more actively in the granular layer, including phospholipids. The increase in phospholipid synthesis in the stratum granulosum, despite the decreased requirement for the formation of most organelle membranes at this strata of the epidermis, could be explained either by: *a)* the possibility that one or more phospholipids may serve as precursors of the sphingolipids emerging during cornification (via degradation of sphingomyelin); *b)* phospholipids may be required to form the large numbers of lamellar bodies that are synthesized in the outer epidermis (26); or *c)* the hypertrophy of the granular cell requires a much larger surface area of plasma membrane (8). Degradation of sphingomyelin results in ceramide formation, which could serve as a source of sphingolipids in the epidermis (27). Yet, our studies did not demonstrate a selective increase of sphingomyelin synthesis (Table 7), consistent with the importance of such a pathway in the epidermis. Lamellar bodies are organelles enriched in phospholipids (26), which secrete their contents at later stages of epidermal differentiation (1, 2). Furthermore, the high rates of phospholipid synthesis in the stratum granulosum may be attributed not only to one or more of the reasons described above, but also to their role as a precursor of free fatty acids, which occur in abundance in the stratum corneum (8, 19, 24). Finally, sphingolipids could be generated from phospholipids via an acyl transferase pathway (28). Although a specific phospholipid-sphingolipid acyl transferase system has not yet been described in the skin, there is evidence for fatty acid recycling during cornification (29).

Our results also demonstrate that the epidermis can synthesize a full spectrum of lipids. We and others have shown that the epidermis has the ability to synthesize certain types of lipids in vivo and in vitro (7, 9, 13). The capacity of the epidermis to synthesize large quantities of sterols (6, 9, 10, 13, 14) is consistent with both the absence of LDL receptors on the membranes of differentiating keratinocytes (11, 14–16) and with the observation that cutaneous sterologenesis is not influenced by circulating cholesterols levels (10). However, since the epidermis apparently lacks the Δ^6 desaturase (17), the enzyme required to generate arachidonic acid from linoleic acid, it must derive at least this fatty acid from the circulation. Thus, it is pertinent that the epidermis has recently been shown to synthesize fatty acids (9). Moreover, both epidermal sterologenesis and fatty acid synthesis are regulated by permeability barrier requirements (6, 19, 18). Whether modulation in the permeability barrier might specifically modulate lipid biosynthesis in the stratum granulosum is currently under investigation.

Finally, the accelerated rates of cholesterol sulfate synthesis in the outer epidermis are consistent with prior studies in epidermis, which showed the highest level of this polar sterol metabolite in the stratum granulosum (30). Cholesterol sulfate, along with other residual polar lipids in the outer epidermis (31), appears to mediate directly the cohesion and desquamation of the stratum corneum (32). But

indirect mechanisms exist for cholesterol sulfate in the regulation of lipogenesis, since this sterol can bypass membrane receptors and directly influence both sterol and glycerolipid metabolism (33, 34).

Dr. Vijay Patel provided excellent technical assistance and Mr. Bil Chapman capably prepared the manuscript. This work was supported by NIH grants AM 19098 and 29908 and the Medical Research Service, Veterans Administration. Drs. Feingold and Williams are recipients of V.A. Clinical Investigator Awards.

Manuscript received 24 August 1987 and in revised form 18 October 1987.

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