Interaction between sphingosine and cholesteryl sulfate in epidermal lipids

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Abstract Free sphingosine, a material with multiple and potent biological activities, is known to occur in high concentration in mammalian epidermis. In the present study, thin-layer chromatography showed that in lipid extracts of human and pig stratum corneum, sphingosine forms a relatively stable compound with endogenous cholesteryl sulfate. NMR spectrometry of sphingosine and its hydrochloride, sulfate, and mixtures with cholesteryl or dodecyl sulfate showed that interaction with the organic sulfates constituted simple salt formation. Under neutral or weakly acidic conditions, such salts were only slightly dissociated and migrated on thin-layer chromatograms as discrete compounds. Thin-layer chromatography revealed undissociated salt formation between several long-chain bases and organic sulfates, and showed that their interaction is stoichiometric. However, undissociated salts were not formed between long-chain bases and fatty acids or phosphatidic acid. Undissociated salt formation may therefore be specific for organic bases and sulfates. It was concluded that the free sphingosine in the stratum corneum may be present as its cholesteryl sulfate salt and in this form be unavailable for permeation into the viable epidermal cells. **—Downing, D. T., R. W. Dose, and W. Abraham.** Interaction between sphingosine and cholesteryl sulfate in epidermal lipids. *J. Lipid Res.* 1993. **34:** 563–569.

Supplementary key words stratum corneum • stearylamine • sulfatide • phosphatidic acid

Sphingosines can act as potent inhibitors of a variety of enzyme systems (1–6) and as mediators of epidermal growth factor receptor activity (7). In some studies, the biochemical activity of the long-chain bases was confined to specific stereoisomers (8), while in other instances a variety of sphingosines (9, 10), lysosphingolipids (11), and even stearylamine (6, 9), showed high activity. Sphingosine has been shown to stimulate cell proliferation (12), while having inhibitory activity against a variety of microorganisms (13).

In view of its broad biological activity (14), it was interesting to find that free sphingosine is present in relatively high concentration in mammalian epidermis (15, 16), especially the stratum corneum, where it appears to be produced by a ceramidase acting on the abundant ceramides in the tissue (17). It was proposed that free sphingosine produced in the stratum corneum may regulate the rate of keratinocyte proliferation in the germinative cells. However, in the present study it was found that the sphingosine in epidermal stratum corneum may largely be sequestered as an undissociated salt with cholesteryl sulfate. This may have implications for the biological activity of sphingosine and for the role of cholesteryl sulfate in epidermal function, and might also help to explain the variable specificity in biological activity that has been reported for long-chain bases.

To elucidate the generality of undissociated salt formation between anionic and cationic lipids, a variety of such mixtures was examined by thin-layer chromatography. In addition, the stoichiometry of the interaction between long-chain bases and organic sulfates was examined by quantitative thin-layer chromatography.

MATERIALS AND METHODS

Lipids

Free sphingosine and dihydrosphingosine, sphingosine hydrochloride and sulfate, stearylamine, stearic acid, and cholesteryl sulfate sodium salt were obtained from Sigma (St. Louis, MO). Cholesteryl sulfate calcium salt was the product of the Nippon Chemical Company. Pure sodium dodecyl sulfate was provided by The Procter & Gamble Co. (Cincinnati, OH). Phosphatidic acid and bovine sulfatide were obtained as their sodium salts from Matreya Inc. (Pleasant Gap, PA). All lipids were dissolved in chloroform–methanol 2:1 for use and for the preparation of mixtures.

Pig stratum corneum lipids were extracted with chloro-

Abbreviations: TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; CS, cholesteryl sulfate; SC, stratum corneum.

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form–methanol from tissue isolated from full thickness epidermis by treatment with trypsin (18). In some instances, inorganic salts and soluble proteins were removed from the pig lipids by extracting a chloroform–methanol solution with 3% acetic acid in water. Additional treatment with 1 M NaOH in 95% methanol at 45°C for 1 h was used to hydrolyze esters in the pig stratum corneum lipids.

Samples of human stratum corneum lipids were obtained by pouring 200 ml of ethanol over each lower leg of volunteers and allowing the extract to collect in a stainless steel basin. The solvent was removed on a rotary evaporator and the residual lipid was suspended in ether and passed through a column of silica gel that had been prewashed with methanol and then ether. Continued elution with ether removed the less polar lipids of predominantly sebaceous origin, and subsequent elution with chloroform–methanol–water 200:100:3 recovered the polar epidermal lipids.

Thin-layer chromatography

All chromatograms were carried out on 0.25-mm-thick layers of silica gel on glass plates (catalog #16330 or 16331, Alltech Associates, Deerfield, IL). The adsorbent was cleaned before use by developing the plates in chloroform–methanol–acetic acid 200:100:3 and then allowing them to dry in air. In most instances the adsorbent was scribed into 6-mm-wide lanes before application of 1–10 μg of the lipid standards or 40–80 μg of epidermal lipid samples.

The chromatograms were usually developed to 10 cm with chloroform–methanol 40:10 containing 1 part of either acetic acid or 36% aqueous ammonium hydroxide, as noted. After drying, the chromatograms of epidermal lipids were redeveloped to 20 cm with chloroform–methanol–acetic acid 190:9:1 to 20 cm. Some chromatograms were simply developed to 20 cm in chloroform–methanol 40:10 containing either acetic acid or ammonia.

For visualization, the chromatograms were sprayed with 50% sulfuric acid and placed on a cold aluminum slab that was then slowly raised to 220°C on a hot plate over a period of 40 min. The location of cholesterol derivatives was confirmed by the initial pink coloration produced under these charring conditions. Slightly later during charring, glycolipids produced transient magenta-colored spots.

After completion of charring, when all of the sulfuric acid had been evaporated and the plates had cooled, quantitation of the chromatograms was obtained with a photodensitometer (model CS-930, Shimadzu Scientific Instruments Inc, Columbia, MD). Electronic correction (linearizer × 2) was used for the inherent nonlinearity of absorbance in light-scattering media (19).

Stoichiometry

To demonstrate stoichiometry of their interaction, 10 mM solutions of dihydrosphingosine and cholesteryl sulfate were prepared in chloroform–methanol 2:1. These solutions were combined in several different ratios. A 1-μl aliquot of each solution was applied in successive lanes of a thin-layer chromatogram and developed to 10 cm with chloroform–methanol–acetic acid 40:10:1. The chromatograms were then charred and quantified by photodensitometry.

An alternative procedure was also used to confirm stoichiometry for the interactions of cholesteryl sulfate and dodecyl sulfate with sphingosine and stearylamine. For this, an aliquot of one binary mixture of these four anion–cation pairs was applied at each corner of a 20 × 20 cm TLC plate that had been divided into four 10-cm squares. Each section was then developed in one direction with chloroform–methanol–acetic acid 40:10:1, and then at right angles with chloroform–methanol–

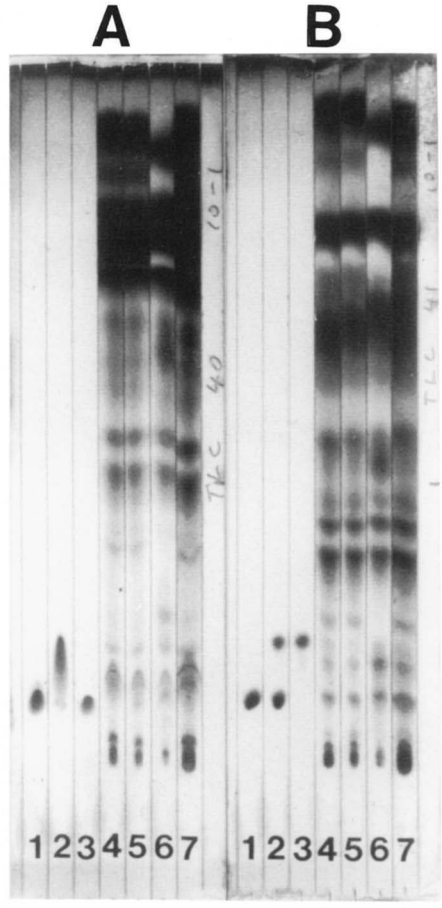

Fig. 1. Chromatograms developed with chloroform–methanol–acetic acid 40:10:1 to 10 cm (A), or with chloroform–methanol–ammonia 40:10:1 to 10 cm (B), followed in both cases by chloroform–methanol–acetic acid 190:9:1 to 20 cm. 1, Cholesteryl sulfate; 2, cholesteryl sulfate + sphingosine; 3, sphingosine; 4, 40 μg pig SC lipids; 5, pig SC lipids (acid washed); 6, pig SC lipids (acid-washed and saponified); 7, 80 μg pig SC lipids.

ammonia 40:10:1. In this way, the salt formed by each mixture was resolved from any excess of either moiety, and in the second direction each salt was resolved into its constituent moieties. The chromatograms were then charred and the lipids were quantified by photodensitometry, using the zig-zag scanning procedure appropriate for misshapen areas. The observed ratio of each pair of salt constituents was then compared with their theoretical ratio, based on the carbon content of each lipid.

Specificity of the interaction

To investigate the specificity of the effect of salt formation on chromatographic mobility, sphingosine, dihydrosphingosine, and stearylamine were each combined with a similar amount of cholesteryl sulfate, dodecyl sulfate, phosphatidic acid, sulfatide, or stearic acid in binary mixtures. Each mixture was subjected to thin-layer chromatography in chloroform–methanol–acetic acid 40:10:1 to observe any effects of salt formation on chromatographic mobility.

NMR

Proton magnetic resonance spectra were obtained using 0.5 mg of lipid, or binary lipid mixtures, in 0.5 ml of 2:1 deuterochloroform–deuteromethanol in a Bruker AMX-600 FT NMR spectrometer.

RESULTS

Interaction between cholesteryl sulfate and sphingosine in epidermal lipids

When subjected to thin-layer chromatography in neutral or weakly acidic solvents, lipids from pig stratum corneum (**Fig. 1A**) or from human skin surface (**Fig. 2A**) usually revealed little material migrating in the regions expected for either cholesteryl sulfate or sphingosine. Instead, two or more overlapping, comet-shaped spots were observed, each of which showed the pink coloration during charring that is characteristic of the cholesterol moiety. These spots migrated ahead of the cholesteryl sulfate standard. On the suspicion that this effect resulted from interaction between cholesteryl sulfate and sphingosine, chromatograms containing these two lipids, separately and as an equimolar mixture of the two, were developed with chloroform–methanol–acetic acid (Figs. 1A and 2A). Cholesteryl sulfate and sphingosine each produced a well-defined circular spot, whereas their mixture produced a more mobile, comet-shaped spot. When the same lipids were chromatographed in chloroform–methanol–ammonia, cholesteryl sulfate and sphingosine each produced discrete spots whether applied singly or as a mixture (Figs. 1B and 2B). Likewise, in the ammoniacal solvent, the pig stratum corneum lipids showed discrete spots in the regions appropriate for cholesteryl sulfate and sphingosine.

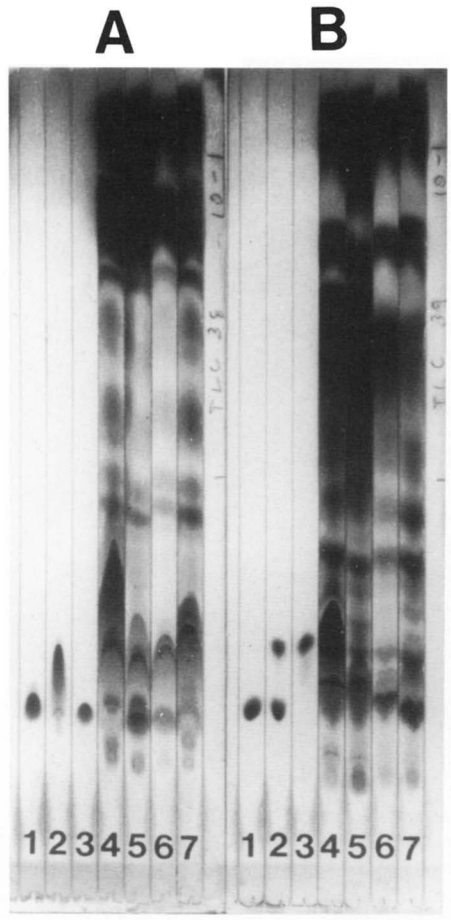

Fig. 2. Chromatograms developed as in Fig. 1. 1, Cholesteryl sulfate; 2, cholesteryl sulfate + sphingosine; 3, sphingosine; 4–7, human skin surface lipids (subjects A–D).

For the human epidermal lipids, the chromatograms were always complicated by contamination, apparently with sulfated detergents such as dodecyl sulfate and dodecylethoxy sulfates. Improved resolution of these ubiquitous contaminants was obtained by developing the chromatograms with chloroform–methanol–ammonia 40:10:1 to 20 cm followed by chloroform–methanol–acetic acid 40:10:1 to 20 cm, as shown in **Fig. 3.** Densitometric quantitation of these chromatograms showed an average 4% cholesterol sulfate in the superficial lipids from 25 subjects, comparable with the level in desquamated human corneocytes (20). An average of 3% sphingosine for the same subjects was much higher than previously reported (15, 16) and requires confirmation by isolation and detailed analysis of the material.

Specificity of interaction between anionic and cationic lipids

Cholesteryl sulfate, dodecyl sulfate, and bovine sulfatides were each found to produce chromatographically resolvable salts from their binary mixtures with sphingosine, dihydrosphingosine, or stearylamine. As with choles-

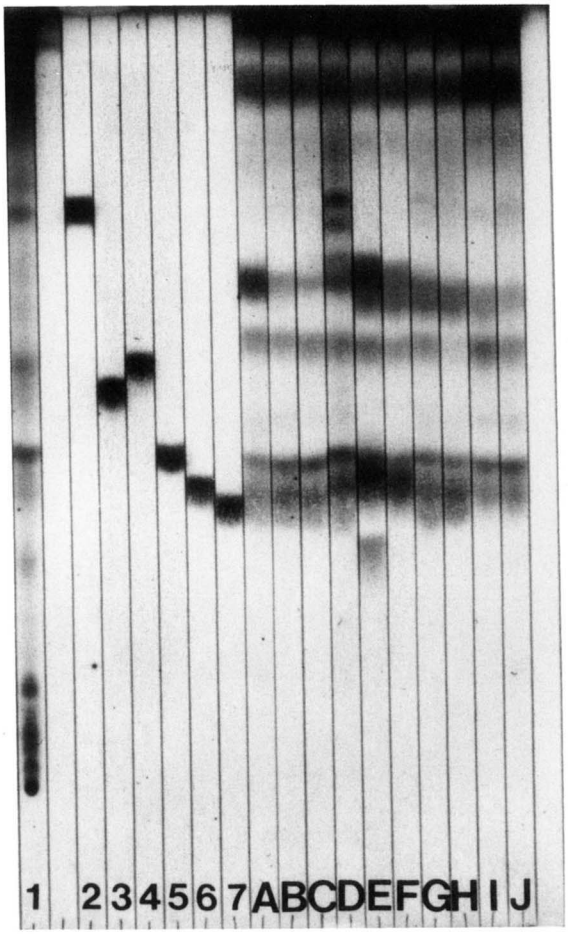

Fig. 3. Chromatograms developed with chloroform–methanol–ammonia 40:10:1 to 20 cm, followed by chloroform–methanol–acetic acid 40:10:1 to 20 cm. 1, Pig stratum corneum lipids; 2, stearylamine; 3, sphingosine; 4, dihydrosphingosine; 5, cholesteryl sulfate; 6, dehydroepiandrosterone sulfate; 7, dodecyl sulfate; A-J, skin surface lipids from a series of human subjects.

teryl sulfate and sphingosine (Figs. 1 and 2), each of these anion–cation pairs produced a more mobile, comet-shaped spot when their chromatograms were developed with the acidic solvent (**Fig. 4A** see also Figs. 5, 6).

Phosphatidic acid required the use of formic acid instead of acetic acid in the solvent mixture to suppress streaking. Under these conditions, mixtures of phosphatidic acid with sphingosine or stearylamine did not produce a discrete spot corresponding with salt formation. However, both the anion and cation in each case showed increased mobility compared with the pure constituents, as though transient salt formation may initially have aided their migration (**Fig. 5**). Salt formation between the organic sulfates and long-chain bases was not changed by the substitution of formic acid for acetic acid in the developing solvent.

No effects on chromatographic mobility were observed as the result of mixing stearic acid with any of the long-chain bases.

Confirmation of salt formation by NMR

The interaction between organic sulfates and sphingosine was confirmed as simple salt formation by the results of NMR spectrometry of sphingosine, its hydrochloride and sulfate, and equimolar mixtures with cholesteryl sulfate and dodecyl sulfate in deuterochloroform–deuteromethanol (**Table 1**). The resonances for individual protons attached to the first three carbons of sphingosine were shifted downfield to virtually identical extents by formation of the hydrochloride, sulfate, cholesteryl sulfate, and dodecyl sulfate salts. This appeared to eliminate the possibility that some specific interaction takes place between sphingosine and the organic sulfates that might have been analogous to ketal formation with the 1,3-hydroxyl system of sphingosine.

Stoichiometry of salt formation

Fig. 6 illustrates the interaction between cholesteryl sulfate and dihydrosphingosine when chromatographed in molar ratios of 2:1, 1.5:1, 1:1, 1:1.5, and 1:2. The colora-

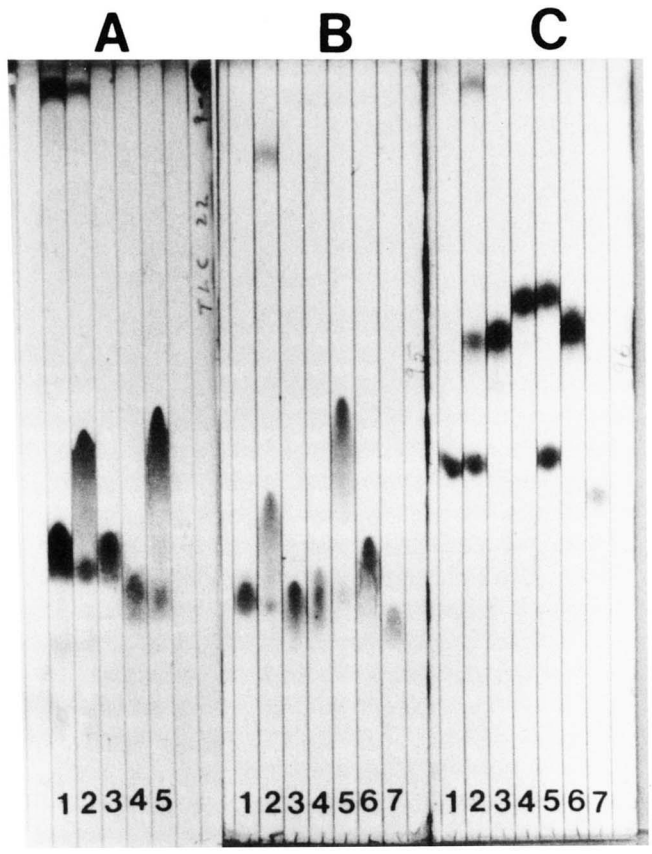

Fig. 4. Chromatograms developed with chloroform–methanol–acetic acid 40:10:1 to 20 cm (A and B) or in chloroform–methanol–ammonia 40:10:1 to 20 cm (C). A: 1, Cholesteryl sulfate; 2, cholesteryl sulfate + sphingosine; 3, sphingosine; 4, sodium dodecyl sulfate; 5, sodium dodecyl sulfate + sphingosine. B and C: 1, Cholesteryl sulfate; 2, cholesteryl sulfate + sphingosine; 3, sphingosine; 4, stearylamine; 5, stearylamine + cholesteryl sulfate; 6, sphingosine sulfate; 7, sodium dodecyl sulfate.

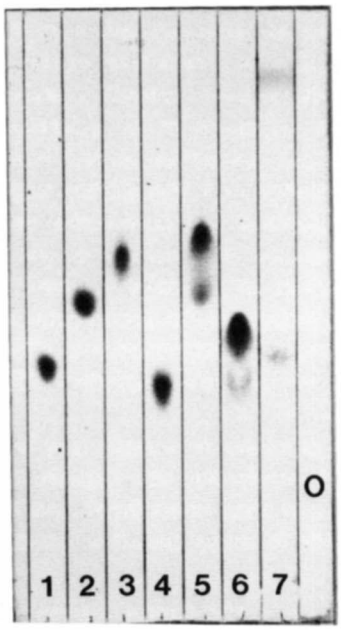

Fig. 5. Chromatograms developed with chloroform–methanol–formic acid 40:10:1 to 10 cm. 1, Dihydrosphingosine; 2, stearylamine; 3, phosphatidic acid; 4, bovine sulfatides; 5, stearylamine + phosphatidic acid; 6, sulfatides + dihydrosphingosine; 7, sphingosine + stearic acid. O indicates the level of the origin.

tion produced during charring showed that there was actually a slightly higher concentration of cholesteryl sulfate than intended. That is, the less mobile spot produced by the intended equimolar mixture (lane 4) showed the presence of free cholesteryl sulfate; in the presence of excess dihydrosphingosine, cholesterol coloration was confined to the more mobile spot (lanes 5 and 6). Nevertheless, photodensitometric quantitation showed reasonable agreement with theoretical ratios between the amounts of adduct and excess constituent in each case (**Table 2**). These calculations were based on the 27 carbons of the cholesteryl moiety and the 18 carbons of sphingosine. It was found that no correction was required for difference in charring between the two moieties, because the equimolar aliquots of the pure constituents that were applied to the chromatograms (lanes 1 and 7) produced relative densitometric peak areas of 59.9:40.1, compared with the theoretical ratio of 60:40 (27:18) for the cholesterol:sphingosine carbons.

In the alternative demonstration of stoichiometry, each chromatographically resolved salt was subsequently separated into its constituents by development in a second direction before charring and quantitation of the chromatograms (**Fig. 7**). The ratios of the densitometric peak areas for the four binary mixtures of cholesteryl sulfate and dodecyl sulfate with sphingosine and stearylamine are compared with their theoretical values in **Table 3.**

TABLE 1. Chemical shifts of some sphingosine derivatives (PPM at 600 MHz)

Lipid	H1A	H1B	H2	H3
Sphingosine	3.47	3.39	2.55	3.79
Sphingosine hydrochloride	3.58	3.50	3.00	4.12
Sphingosine sulfate	3.57	3.49	3.00	4.12
Sphingosine/cholesteryl sulfate	3.55	3.49	2.98	4.10
Sphingosine/dodecyl sulfate	3.58	3.50	2.96	4.10

DISCUSSION

Effects on chromatographic mobility produced by the interaction between organic sulfates and amines appear to result from the formation of weakly dissociated salts that have greater chromatographic mobility than their respective moieties. The effect is weak for phosphatidic acid and absent for stearic acid, presumably because of the greater degree of dissociation of their amine salts. However, demonstration of the effect may also be influenced by differences in the relative mobilities of the ion pairs, which would be more readily resolved as their mobilities became more dissimilar. Therefore, the effect of salt formation is likely to be most apparent where the anion and cation have similar mobilities, as is the case with the long-chain amines and lipid sulfates.

The significance of these observations lies first in avoiding interference with the analysis of lipid mixtures containing interacting substances. This applies most particularly to the analysis of epidermal lipids, where cholesteryl sulfate and sphingosine are quantitatively (and perhaps

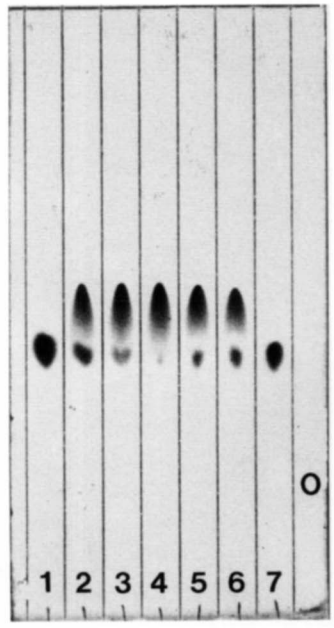

Fig. 6. Chromatograms developed with chloroform–methanol–acetic acid 40:10:1 to 10 cm. 1, Cholesteryl sulfate (CS); 2, CS + dihydrosphingosine (DHS) 2:1; 3, CS + DHS 1.5:1; 4, CS + DHS 1:1; 5, CS + DHS 1:1.5; 6, CS + DHS 1:2; 7, DHS. O indicates the level of the origin.

TABLE 2. Quantitative TLC analysis of salt formation with varying ratios of cholesteryl sulfate (CS) and dihydrosphingosine (DHS) applied to chromatograms

	Lane						
	1	2	3	4	5	6	7
	Amounts Applied to Chromatograms (*nmol*)						
CS	10.0	6.66	6.0	5.0	4.0	3.33	0.0
DHS	0.0	3.33	4.0	5.0	6.0	6.66	10.0
	Theoretical Peak Ratios						
Salt		62.5	76.9	100.0	83.3	71.4	
Excess CS		37.5	23.1				
Excess DHS					16.7	28.7	
	Peak Ratios Found						
Salt		65.5	81.6	95.1	78.7	69.1	
Excess CS		34.5	18.4	4.9			
Excess DHS					21.3	30.9	

biologically) significant constituents. For the analysis (and perhaps the function) of human skin lipids, the observed amine–sulfate interactions may have further significance because of the ubiquitous presence of sulfated detergents.

Regarding potential biological significance, it may be inferred that in native stratum corneum, where a high proportion of free fatty acids is present and the pH is slightly acidic, there likewise may be only slight dissociation of the sphingosine/cholesteryl sulfate salt. As a result, free sphingosine in the stratum corneum may be effectively sequestered in the intercellular lipid bilayers, limiting its partition into the aqueous milieu of the viable cells. This may explain how a high level of free sphingosine is able to exist in the stratum corneum without obvious toxic effects on the living layers of the epidermis.

Sphingosine salt formation in the stratum corneum may also be invoked in a speculation regarding homeostasis of the epidermis. It is known that desquamation of corneocytes from the skin surface occurs concomitantly with the hydrolysis of cholesteryl sulfate (20, 21). This could release sphingosine for permeation into the viable cells, stimulating their proliferation by effects on the germinative cells (12), and promoting their differentiation by mobilizing calcium (11).

Sphingosine salt formation might also be invoked to explain some of the conflicting observations regarding structural specificity of long chain bases and their biological activity (8, 9, 13). If endogenous free sphingosine in a biological system is largely in the form of undissociated organic salts, and perhaps as a result is strongly sequestered in membranes, the addition of any long-chain base could displace endogenous sphingosine to produce its specific biological effects. The differing results of investigations into the structural specificity of sphingosines and stearylamine for biological activity may simply reflect the degree to which sphingosine salts are present in the systems. A variety of endogenous and exogenous sulfates, including cholesteryl sulfate, sulfatides and sulfated detergents, might affect the biochemical activity of sphingosine.

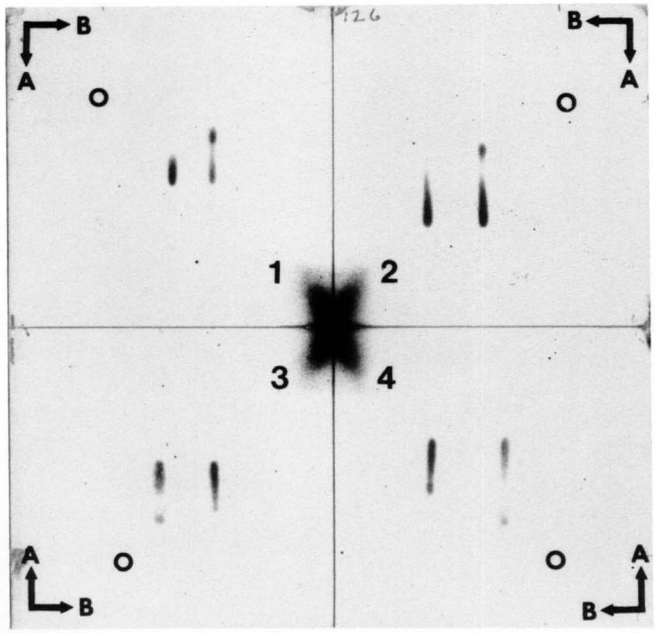

Fig. 7. Two-dimensional chromatograms developed in direction A with chloroform–methanol–acetic acid 40:10:1 and then in direction B with chloroform–methanol–ammonia 40:10:1. 1, Cholesteryl sulfate/sphingosine; 2, cholesteryl sulfate/stearylamine; 3, dodecyl sulfate/sphingosine; 4, dodecyl sulfate/stearylamine. O indicates the positions of the origins.

TABLE 3. Quantitative TLC analysis of resolved salt compositions

Mixture	Number of Carbons	Theoretical Ratio	Ratio Found
1. Cholesteryl sulfate	27	60	62.4
Sphingosine	18	40	37.6
2. Cholesteryl sulfate	27	60	52.3
Stearylamine	18	40	47.7
3. Dodecyl sulfate	12	40	44.0
Sphingosine	18	60	56.0
4. Dodecyl sulfate	12	40	37.5
Stearylamine	18	60	62.5

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