Cholesteryl sulfate: the major polar lipid of horse hoof

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Abstract The lipids of horse hoof have been analyzed by quantitative thin-layer chromatography. The major components include cholesterol (37–40%), six groups of ceramides (10–15%), and cholesteryl sulfate (15–20%). Free fatty acids are abundant (15.8%) in the outer fully keratinized hoof, but are present at only low levels (3.1%) in the softer hyponychium. The material identified as cholesteryl sulfate was isolated by preparative thin-layer chromatography and characterized by a combination of chemical, chromatographic, and spectroscopic methods. The infrared spectrum of the isolated material had absorption bands at 800, 1063, 1200, and 1235 cm^{-1}, indicating a sulfate ester. This sulfolipid was nonsaponifiable, but upon acid hydrolysis yielded cholesterol as the only charrable product, which was identified by its chromatographic behavior and by its electron impact mass spectrum. The isolated sulfolipid also had the same mobility on thin-layer chromatography as authentic cholesteryl sulfate in several different solvent systems. Sulfated gangliosides, which were previously reported to be major horse hoof lipids, were not found among the principal lipid components in the present study. It is concluded that cholesteryl sulfate is the major polar lipid of horse hoof. This may be a significant factor determining the high degree of cohesiveness of this fully keratinized tissue.—**Wertz, P. W., and D. T. Downing.** Cholesteryl sulfate: the major polar lipid of horse hoof. *J. Lipid Res.* 1984. **25:** 1320–1323.

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The outermost, fully keratinized layers of the mammalian stratum corneum provide an effective barrier to water loss and protect the underlying tissues against a variety of environmental insults (1). It is currently accepted that the water barrier function resides in the multiple extracellular membranes which are principally composed of lipids (1–4). These extracellular structures, which arise from the contents of epidermal lamellar granules (5), were first detected by Breathnach et al. (2) and were subsequently shown by Squier (3) to retard the movement of water-soluble tracers. Thus, lipids appear to be responsible for the barrier to transepidermal water loss.

There also have been indications that stratum corneum lipid may be one of the factors governing the orderly cohesion-dishesion behavior of the epidermal keratinocytes. The most compelling evidence in this area has come from studies of recessive X-linked ichthyosis, where the only known biochemical defect is an absence of sterol sulfatase (6). This enzyme deficiency results in the accumulation of cholesteryl sulfate in the stratum corneum (7, 8), and the normal orderly desquamation of individual keratinocytes does not occur. Rather, clusters of horny cells remain partially adherent, and this results in the characteristic scaly appearance (8). Additional evidence is provided by the studies of Smith and colleagues (9) who have been able to reaggregate individual, delipidized corneocytes into sheets resembling native stratum corneum by the addition of stratum corneum lipids. These reconstituted sheets have viscoelastic properties and water barrier properties similar to freshly isolated stratum corneum.

In spite of the importance of lipids for the normal structure and function of the stratum corneum, the structures of the epidermal lipids have not yet been completely defined. Gray and associates (10–15) made considerable progress at identifying the general lipid classes found at various depths within the epidermis. However, only recently have the precise structures for the major epidermal sphingolipids been determined (16–19).

Although the major lipids of the stratum corneum consist of cholesterol, free fatty acids, and ceramides (10, 15), small amounts of triglycerides and more polar species have also been reported (10). Among the most polar lipids of the stratum corneum are cholesteryl sulfate (7, 10, 20) and an unusual ganglioside sulfate which has been called ungulic acid (21, 22). Ungulic acid has been reported to occur in human epidermis, hair, nails, and kidney, as well as horse hoof (21, 22),

Abbreviation: TLC, thin-layer chromatography.

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where it was said to account for nearly 50% of the total lipid (21).

The identification of ungulic acid has been questioned by Ueta et al. (23). These investigators found the major lipid in cow hoof to be cholesteryl sulfate. No ganglioside sulfates were noted in this tissue. However, although horses and cows are both ungulates, this relationship does not guarantee similar lipid compositions for their hoofs or other tissues. In fact, the sebaceous lipids from the skins of horses (24) and cows (25) have been analyzed and are not at all similar; therefore, the apparent absence of ungulic acid from cow hoof does not negate the identification of ungulic acid in horse hoof or other tissues.

In order to resolve this problem, the lipids of horse hoof have been reinvestigated. The major polar lipid from horse hoof has been identified on the basis of its chemical and chromatographic properties, infrared absorption spectrum, and electron impact mass spectrum. As in the case of bovine hoof (23), this lipid has proved to be cholesteryl sulfate, not a ganglioside sulfate.

METHODS AND RESULTS

Fresh trimmings from horse hooves were obtained from a local farrier. The hoof material was rinsed under tap water to reduce surface contamination and then dried thoroughly in vacuo. The dried hoof was reduced to powder with a steel rasp, and the powdered hoof was then extracted at room temperature for successive 1-hr intervals with chloroform–methanol 2:1, 1:1, and 1:2. The extracts were combined and the solvent was evaporated under a stream of nitrogen. The soft inner wall lining the hoof (hyponychium) was ground and extracted separately from the hoof proper. The yield of lipid was 1.5 and 1.9% on a dry weight basis for the hoof and hyponychium, respectively.

The hoof lipids were redissolved at a concentration of 50 mg per ml of chloroform–methanol 1:1, and examined by quantitative thin-layer chromatography (TLC). The nonpolar lipids were resolved by successive developments with hexane, and hexane–toluene 1:1, followed by two developments half-way up the plate with hexane–ethyl ether–acetic acid 80:20:1. Standard materials included commercially available cholesterol, palmitic acid, triolein, cholesteryl oleate, and squalene (Sigma Chemical Co., St. Louis, MO). Wax diesters and giant ring lactones were identified by reference to the materials previously isolated from horse sebum (24). After development and air drying, the chromatographic plate was sprayed with 50% sulfuric acid, charred on a 220° hot plate, and the charred lipids were quantitated by densitometry as previously described (26, 27). Ceramides, which remain on the origin after the above development regimen, were resolved in subsequent chromatograms by two developments with chloroform–methanol–acetic acid 190:9:1. The ceramides were identified by comparison with epidermal ceramides prepared from pig skin (18). The most polar lipids were resolved in further chromatograms by development with chloroform–methanol–water 40:10:1, as shown in **Fig. 1.** This group of lipids consists of several unidentified minor components and one major spot that had the same TLC mobility as authentic cholesteryl sulfate (Steraloids, Inc., Wilton, NH). Upon charring with sulfuric acid, both standard cholesteryl sulfate and the materials isolated from horse hoof and hyponychium produced a characteristic red-violet color prior to turning black. The materials from both the inner and outer hoof also comigrated with authentic cholesteryl sulfate on silicic acid TLC in a variety of different neutral, acidic, and basic solvent systems having different polarities. These results are summarized in **Table 1**.

The identity of cholesteryl sulfate was determined by a combination of chemical, chromatographic, and spectroscopic methods. For this, the major polar lipid was isolated from both hoof and hyponychium by preparative TLC on a 0.5-mm-thick layer of silica gel 60H. The plates were developed with chloroform–methanol–water 40:10:1. Material with the mobility of cholesteryl sulfate was detected under ultraviolet light after spraying the plate with the fluorescent indicator (8-hydroxy-1,3,6-pyrenetrisulfonic acid sodium salt) and the lipid was eluted from the silica gel with chloroform–methanol 1:1.

The infrared spectrum of the suspected cholesteryl sulfate from horse hoof and hyponychium had absorptions at 800, 1063, 1200, and 1235 cm^{-1}, indicating the presence of a sulfate ester (28).

The purified lipid was found to be unreactive during saponification with 1 N methanolic KOH at 60°C for

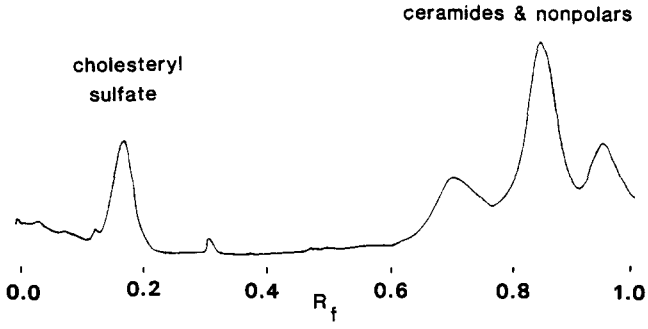

Fig. 1. Thin-layer chromatographic profile of horse hoof lipids. The silicic acid TLC plate was developed with chloroform–methanol–water 40:10:1. After spraying with 50% H_2SO_4, the plate was charred at 220°C and the charred lipids were scanned by photodensitometry.

TABLE 1. Thin-layer chromatographic behavior of the major polar lipid from horse hoof

Solvent System	R_f for Hoof Lipid and Cholesteryl Sulfate
Chloroform–methanol–water	
40:10:1	0.14
24:7:1	0.26
Chloroform–methanol–acetic acid–water	
40:10:1:1	0.23
40:20:1:1	0.78
Chloroform–methanol–3.75 M NH_3 (aq)	
50:25:4	0.52

1 hr. On the other hand, after hydrolysis with 1 N HCl in methanol containing 20 M water at 65°C for 18 hrs, only one charrable material was produced from each sample, and this had the same mobility on TLC as cholesterol. This hydrolysis product also cochromatographed with authentic cholesterol in gas–liquid chromatography on a 6-ft column packed with 3% OV-101 on 80/100 mesh Supelcoport (Supelco, Inc., Bellefonte, PA).

The identification of cholesterol from hydrolyzed cholesteryl sulfate was supported by the electron impact mass spectrum of the acetylated material. This mass spectrum, obtained on a Finnigan 1015 mass spectrometer, was essentially identical to that of standard cholesteryl acetate recorded under the same conditions. A direct probe electron impact mass spectrum of cholesteryl sulfate isolated from hoof was also recorded. This spectrum did not show a molecular ion but included fragments at m/z 386, 370, 368, 353, 301, 275, 260, 255, 247, 213, 199, 185, 173, 171 as major high m/z ions.

The compositions of the two horse hoof samples, as determined by quantitative thin-layer chromatography, are presented in **Table 2.** Also summarized in Table 2 is the lipid composition previously reported for cow hoof (23). As can be seen, free sterol, which probably is mostly cholesterol, is the major lipid component of horse hoof, hyponychium, and cow hoof. Free fatty acids are also an abundant lipid group in hoof proper, where they constitute 15.8% of the total horse hoof lipid and 24% of the cow hoof lipid, but they are not a prominent component in the inner lining of the hoof. The remaining nonpolar lipids found in the present study of horse hoof include triglycerides, wax diesters, lactones, cholesteryl esters, squalene, and hydrocarbons. Of these, the lactones, steryl esters, and wax diesters are probably of sebaceous rather than epidermal origin (24), and the hydrocarbons are likely exogenous. The polar lipids from both horse and cow hoof include 7–15% ceramides and 10–20% cholesteryl sulfate. The unidentified materials from horse hoof include several components of polarity similar to cholesteryl sulfate and, in hyponychium, a somewhat less polar steroidal derivative was also noted.

DISCUSSION

In the present study, horse hoof lipids have been analyzed by quantitative TLC, and the major polar lipid has been characterized in greater detail. This material, which represents 15–20% of the total lipid, does contain a sulfate ester as judged by its infrared spectrum. As reported for ungulic acid (21), this sulfolipid is nonsaponifiable and undergoes a color reaction prior to charring in the presence of sulfuric acid. It also has chromatographic properties similar to those reported for ungulic acid. For example, in the solvent system, chloroform–methanol–water 24:7:1, ungulic acid was reported to have an R_f of 0.31 (21) while the major polar lipid found in the present study had an R_f of 0.26. However, in the present study, acid hydrolysis of this material yielded cholesterol as the only lipid product. Furthermore, the unhydrolyzed hoof lipid behaved exactly like authentic cholesteryl sulfate in a variety of neutral, acidic and basic solvent systems of different polarities (Table 2). It is concluded that cholesteryl sulfate, not a ganglioside sulfate, is the major polar lipid of horse hoof. These results as well as the general lipid composition found for horse hoof are similar to the results obtained by Ueta et al. for cow hoof (23).

The reason for the difference between these results and the earlier work of Leikola, Nieminen, and Teppo (21, 22) is uncertain; however, the earlier workers noted a poor recovery of the lipid during preparative TLC and recrystallization (21). It is possible that the recrystallized material which was analyzed in detail represents

TABLE 2. Composition of hoof lipids

Lipid	Horse Hoof	Horse Hyponychium	Cow Hoof[b]
Cholesterol	36.8[a]	39.8[a]	31
Fatty acids	15.8	3.1	24
Triglycerides	3.2	0.1	14
Wax diesters	tr	0.9	
Lactones	0.4	0.4	
Cholesteryl esters	1.6	4.8	
Squalene	1.4	11.7	
Hydrocarbons	8.8	0.2	
Ceramide 1	0.1	0.3	
Ceramide 2	6.8	8.2	
Ceramide 3	1.4	1.2	
Ceramide 4	0.5	1.1	
Ceramide 5	0.9	1.3	
Ceramide 6	0.3	2.6	
Total ceramides	10.0	13.7	7
Cholesteryl sulfate	19.6	15.3	10
Unidentified	2.3	9.0	4

[a] Weights percent were determined by quantitative TLC.
[b] Taken from Ueta et al. (23). Glucosylceramide (6%) and diglyceride (3%) were also reported.

one of the minor polar lipids rather than the major material revealed by TLC.

The presence of such a high proportion of cholesteryl sulfate in a fully keratinized tissue may be of functional significance. Ueta et al. (23) suggested that cholesteryl sulfate may play a role in the excretion of cholesterol; however, more recent evidence has implicated this lipid in the cohesive properties of epidermal keratinocytes (6–8). Based upon these observations, it has been suggested that cholesteryl sulfate may cement together horny cells, possibly through the formation of complexes with divalent cations (8, 29, 30). Within the context of this hypothesis, it is appropriate that a fully keratinized tissue such as hoof, from which cells are not normally shed, should have a high cholesteryl sulfate level.

In an earlier survey of the lipids of keratinized tissues, several nonsaponifiable materials of polarity similar to cholesteryl sulfate were noted in hair, hoof, horn, and feathers (31). At the time of that survey, the major polar lipid of hoof was taken to be a ganglioside sulfate. In view of the present findings, these data may need to be reevaluated. In any case, a number of these fully keratinized tissues contained substantial amounts of several materials similar in polarity to cholesteryl sulfate, and careful analysis of the lipids from each source is needed before any general conclusions can be drawn.

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