# Stratum Corneum Lipids in Disorders of Cornification

## INCREASED CHOLESTEROL SULFATE CONTENT OF STRATUM CORNEUM IN RECESSIVE X-LINKED ICHTHYOSIS

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ABSTRACT Activity of the microsomal enzyme, steroid sulfatase, is absent in keratinocytes, fibroblasts, and leukocytes of patients with recessive x-linked ichthyosis. This study was undertaken to determine if cholesterol sulfate, a substrate of this enzyme, accumulates in the pathological scale of these patients. Scales from 8 patients with recessive x-linked ichthyosis, 10 patients with other forms of ichthyosis, and normal human outer stratum corneum were extracted with chloroform/water (1:2:0.8 by vol) and lipids were fractionated by quantitative, sequential thin-layer chromatography. Cholesterol sulfate was identified by cochromatography in several solvent systems, by its staining characteristics, by biochemical analysis, and by mass spectrometry. The mean cholesterol sulfate content of recessive x-linked ichthyotic scale was 12.5±0.8% of the total lipid, a fivefold increase over normal ($P < 0.0025$), whereas the cholesterol sulfate content of other ichthyotic scale was normal. This increase in cholesterol sulfate content was accompanied by a decrease in total neutral lipids ($P < 0.0025$) and free sterols ($P < 0.025$) but no change in sterol esters or total sterols. These results demonstrate that deficiency of steroid sulfatase in recessive x-linked ichthyosis results in excessive accumulation of a substrate, cholesterol sulfate, in the pathologic scale, which may underly the pathogenesis of the scaling in this disorder. Measurement of cholesterol sulfate content in scale provides an alternative method to enzymatic assay for the diagnosis of this form of ichthyosis.

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## INTRODUCTION

The ichthyoses comprise a group of dermatoses characterized by increased stratum corneum thickness manifested by excessive scaling. Currently, four major types are distinguished by their clinical features and mode of inheritance (1, 2). Recently, one form, recessive x-linked ichthyosis (RXLI),[1] was found by Shapiro et al. (3) to be associated with a deficiency of the microsomal enzyme, steroid sulfatase (arylsulfatase C), in cultured fibroblasts. More recently, the enzyme has also been found to be lacking in stratum corneum (4), hair bulbs (4), cultured keratinocytes (5), and leukocytes (6). Since deficiency of fibroblast steroid sulfatase has now been confirmed in a large number of patients from many parts of the world (7, 8), Shapiro has proposed that RXLI is due to absence of this enzyme. However, direct evidence for a link between steroid sulfatase deficiency and the pathogenesis of RXLI is still lacking.

Several lines of evidence suggest that lipids are important for normal stratum corneum structure and function. Lipids constitute 6–10% of normal stratum corneum by weight (9, 10), where they are found primarily in the intercellular spaces (11). By ultrastructural and histochemical criteria they form broad, laminated sheets (12), and account for a substantial volume of the normal stratum corneum (13). Numerous studies from humans and experimental animals indicate a critical role for stratum corneum lipids in epi-

[1] *Abbreviations used in this paper*: NHS-SC, normal human sterols-stratum corneum; RXLI, recessive x-linked ichthyosis; TLC, thin-layer chromatography.


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dermal permeability function (cf. 12, 14). Preliminary evidence also suggests that stratum corneum lipids may be essential for the invisible, orderly process of normal desquamation (15, 16).

These studies were undertaken to determine if steroid sulfatase deficiency in RXLI is associated with alterations in stratum corneum lipid composition that might, in turn, underlie the increased scaling in this disorder. Using sequential, quantitative thin-layer chromatography, we have demonstrated a marked, disease-specific increase in cholesterol sulfate content of RXLI scales.

## METHODS

*Patient selection and samples.* Eight patients with RXLI, four patients with ichthyosis vulgaris, four with lamellar ichthyosis, and two with epidermolytic hyperkeratosis were studied. The diagnosis of all patients in this study was established by standard clinical and histological criteria (2). Moreover, the diagnosis of RXLI was confirmed by prior assay of steroid sulfatase activity in fibroblasts and /or leukocytes (6) (Table I).

Scales were obtained from patients with various forms of ichthyosis by gentle scraping with a number 15 scalpel blade. The lower back and/or legs were chosen to minimize contamination with endogenous sebaceous lipids and were left untreated topically for at least 4 wk before scale collection. Normal human outer stratum corneum (NHS-SC) was obtained from desquamating scales after the removal of orthopedic extremity casts (patients 19 and 20) and untreated postsunburn exfoliation (patients 21 and 22).

*Lipid extraction.* Hairs were removed manually from scales and the scales were stored at −20°C until use. Weights were obtained before and after 24 h of vacuum dessication over Drie-Rite. To extract the lipids, scales were soaked overnight in Bligh-Dyer solution (chloroform/methanol/water; 1.0:2:0.8, by vol) (10 mg tissue/ml solvent), and the solvent decanted through a prerinsed Whatman 43 filter (Whatman, Inc., Clifton, N. J.) The cellular debris was again suspended in Bligh-Dyer solution, pulverized in a ground-glass homogenizer, shaken for 30 min on a Burrel wrist-action shaker (Burrell Corp., Pittsburgh, Pa.), centrifuged for 10 min at 2,000 rpm, and filtered. The lipids were then recovered from the solvent as follows. To each 7.6 ml of Bligh-Dyer solution, 2 ml water and 2 ml chloroform were added to split the phases. The lipid-containing infranatant was washed with the aqueous phase of chloroform/methanol/water (4:4:3.6, by vol), and the chloroform phases were pooled, dried under nitrogen, and weighed. All aqueous phases from the extraction procedure also were pooled, dried in a rotary evaporator, resuspended in methanol, chromatographed (see below), extracted, dried under nitrogen, and stored at −4°C in benzene for determination of cholesterol sulfate content by the Ham method (17).

*Thin-layer chromatography.* The lipid extracts were fractionated by quantitative, sequential silica gel thin-layer chromatography (TLC). A known quantity (generally 2–4 mg) of lipid was dissolved in 100–200 μl of chloroform/methanol

TABLE I
*Summary of Clinical Data*

| Patient | Diagnosis | Sex/age | Steroid sulfatase assay (Fibroblasts/Leukocytes) |
|---|---|---|---|
| 1 | RXLI | M/34 | −/− |
| 2 | RXLI | M/14 | ND/− |
| 3 | RXLI | M/23 | ND/− |
| 4 | RXLI | M/31 | −/− |
| 5 | RXLI | M/29 | ND/− |
| 6 | RXLI | M/79 | ND/− |
| 7 | RXLI | M/20 | ND/− |
| 8 | RXLI | M/50 | ND/− |
| 9 | IV | M/23 | ND/+ |
| 10 | IV | F/70 | ND/ND |
| 11 | IV | M/30 | ND/+ |
| 12 | IV | M/70 | ND/ND |
| 13 | LI | F/10 | ND/ND |
| 14 | LI | F/12 | ND/ND |
| 15 | LI | F/18 | ND/ND |
| 16 | LI | F/30 | ND/ND |
| 17 | EHK | M/1 | ND/ND |
| 18 | EHK | F/10 | ND/ND |
| 19 | NHS-SC (cast) | M(3 pooled)/45–60 | ND/ND |
| 20 | NHS-SC (cast) | M/40 | ND/ND |
| 21 | NHS-SC (sunburn) | M/40 | ND/ND |
| 22 | NHS-SC (sunburn) | F/37 | ND/ND |

Abbreviations: IV, ichthyosis vulgaris; LI, lamellar ichthyosis; EHK, epidermolytic hyperkeratosis; M, male; F, female.
−, No appreciable activity; +, activity present; ND, not done.

(1:1, by vol) and applied to commercial silica gel 60 high pressure (HP)-TLC plates (MC/B, Inc., Cincinnati, Ohio) that had been precleaned in chloroform/methanol/glacial acetic acid/water (60:35:0.5:1.5, by vol) and preactivated at 100°C. The first solvent system, tetrahydrofuran/methylal/methanol/4 M ammonium hydroxide (60:30:10:4, by vol), separated cholesterol sulfate from polar and neutral lipids. After development in this solvent, the plates were briefly air dried, sprayed with 1% 8-anilino-1-naphthalene in water, and photographed under black-light fluorescence. The neutral and polar lipid bands were excised and the lipid-containing gel placed in screwcap test tubes with 7.6 ml Bligh-Dyer solution, shaken for 10 min, and centrifuged at 2,000 rpm for 10 min. The solvent was decanted and the gel extracted with solvent a second time. The phases were split and washed, as described above for tissue lipid extraction, and the lipid-containing infranatants dried and transferred in warmed ethanol to minicentrifuge tubes. The lipids were again gently dried under nitrogen, resuspended in 500 μl of ethanol, centrifuged for 45–60 min in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) to sediment any remaining gel, and 50-μl aliquots were removed, dried and weighed on an electrobalance (Cahn Instruments Div., Ventron Corp., Cerritos, Calif., model 21, automatic electrobalance). Initially, the cholesterol sulfate band was extracted with Bligh-Dyer solution, and the aqueous phases saved for monitoring the cholesterol sulfate content (17). Later, the extraction procedure was modified by the addition of 0.1 M KCl to retain the cholesterol sulfate in the chloroform phase. Inclusion of KCl during extraction results in 93.0% recovery in the chloroform phase of a known quantity of cholesterol sulfate, and an additional 5.4, 0.4, and 0.3% are obtained by a second, third, and fourth wash, respectively. However, since retention of some KCl in the chloroform phase resulted in spurious weights, the cholesterol sulfate content was measured colorimetrically (all data presented in this paper are derived from the colorimetric method); we have previously shown that retained KCl does not interfere with this method (B. E. Brown and P. M. Elias, unpublished observations).

The assignment of the abnormal band to cholesterol sulfate is based upon several criteria (Table II) including, (*a*) cochromatography with the authentic compound in three solvent systems; (*b*) characteristic color change upon sulfuric acid treatment (18); (*c*) characteristic mass spectrum; and (*d*) generation of pure cholesterol alone on mass spectrum after solvolysis. After solvolysis only cholesterol was present on mass spectrometry; no 7-dehydrocholesterol or cholecalciferol (vitamin $D_3$) was present.

The pooled and weighed neutral lipid fraction from the previous TLC was further fractionated by silica gel TLC that used petroleum ether/diethyl ether/glacial acetic acid (80:20:1, by vol). These plates were developed and photographed, and the lipids extracted and weighed as described above. This system fractionated the neutral lipids into glycosphingo-lipids, free fatty acids, free sterols, triglycerides, a band containing sterol esters, wax esters, and hydrocarbons, as well as a few unknown bands (5–10% total lipid). The sterol and wax esters were separated from hydrocarbons by development in petroleum ether/diethyl ether (95:5, by vol), extracted and hydrolyzed with 12% boron trichloride, rechromatographed in the neutral lipid solvent, and the free sterol band extracted and weighed.

## RESULTS

Lipid extracts of scale were obtained from 8 patients with RXLI, 10 patients with other forms of ichthyosis, and 4 normal controls. The mean total lipid content of all ichthyotic scales, 12.1±1.3%, (range, 4.3 to 24.1%) did not differ significantly from that of normal human outer stratum corneum (10.5±3.9% (range, 4.4 to 13.3%) (Table III). These totals reflect the sum of the lipid recovered in the organic solvent phase plus the cholesterol sulfate recovered from the aqueous phase (Table III).

Using the tetrahydrofuran/methylal/methanol/ammonium hydroxide solvent system (Methods), cholesterol sulfate fractionated as a single band distinct from polar lipids, neutral lipids, and glycosphingolipids. A prominent cholesterol sulfate band was encountered only in RXLI, whereas neither normals nor patients

TABLE II
*Criteria for Identification of Cholesterol Sulfate*

A. Thin-layer chromatography—cochromatography with authentic compound in:
   1. Tetrahydrofuran:methylal:methanol:4 M ammonium hydroxide (60:30:10:4)
   2. Chloroform/methanol/water/acetic acid (60:35:4.5:0.5)
   3. a) Chloroform/methanol/water (90:10:1)
      b) Petroleum ether/diethyl ether/acetic acid (70:50:1)

B. Colorimetric
   1. Identification as sterol by Ham method (17)
   2. Color reaction (reddish-purple) with 50% sulfuric acid spray (18)

C. Mass spectrometry
   1. Cholesterol sulfate identified by field atomic bombardment (FAB) (40).
   2. Cholesterol identified after solvolysis.

TABLE III
*Total Lipid Extracted from Ichthyotic Scales vs. NHS-SC*

| Disease | Mean lipid weight percent (% total weight)±SEM | | |
|---|---|---|---|
| | Chloroform phase | Aqueous phase* | Total |
| RXLI ($n$ = 8) | 10.8±1.3 | 0.7±0.2 | 11.2±1.3 |
| Ichthyosis vulgaris ($n$ = 4) | 13.7±4.4 | 0.1±0.0 | 13.7±4.4 |
| Lamellar ichthyosis ($n$ = 4) | 13.2±3.4 | <0.1 | 13.2±3.4 |
| Epidermolytic hyperkeratosis ($n$ = 2) | 10.7 | 0.1 | 10.8 |
| Normal ($n$ = 4) | 9.6±2.5 | 1.1±0.6 | 10.5±3.9 |

* The only lipid encountered on thin-layer chromatography of the aqueous phase was cholesterol sulfate; quantitation done colorimetrically (Methods).

with other forms of ichthyosis displayed a prominent fraction of comparable mobility (Figs. 1 and 2).

Cholesterol sulfate was recovered from both the aqueous and chloroform phases of the extraction procedure (see Table IV). The proportion of cholesterol sulfate in RXLI lost into the aqueous phase varied between 1.1 and 6.6% (mean, 2.6±0.1) of the total lipid, and constituted as much as 40% of the total cholesterol sulfate recovered. The substantial loss of cholesterol sulfate into the aqueous phase underlined the importance of assaying this compartment in any attempt to quantitate tissue cholesterol sulfate concentrations. Whereas the mean total cholesterol sulfate content of RXLI scale was 12.5±0.8% of the total lipid, normal human stratum corneum lipids contain only 2.6±0.2% cholesterol sulfate. This represents a mean increase in cholesterol sulfate content five times over normal. The cholesterol sulfate content of other ichthyotic scale was normal (lamellar ichthyosis: mean, 1.5±0.2%; epidermolytic hyperkeratosis: 2.4%; ichthyosis vulgaris: 2.9±0.7%); only patient 10 with ichthyosis vulgaris had a cholesterol sulfate content (5.1%) approaching the lower limits of that observed in RXLI (range, 7.0 to 17.9%).

Polar lipids constituted a minor fraction of RXLI scale lipids (mean, 2.9±0.2%) and normal human sterols-stratum corneum (NHS-SC) (mean, 1.6±0.1%): Total neutral lipids in RXLI are significantly reduced

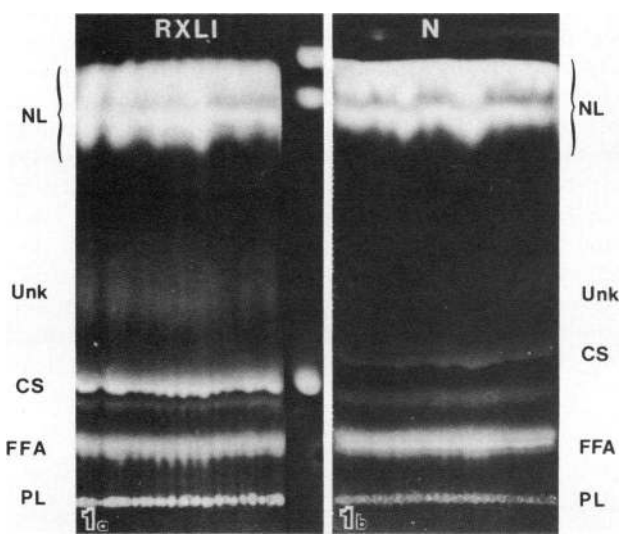


FIGURE 1 Cholesterol sulfate thin layer chromatography of recessive x-linked ichthyosis scale (1a) vs. NHS-SC (1b). In this solvent system, cholesterol sulfate migrates in an intermediate position, behind the neutral lipids and in front of free fatty acids and polar lipids. A more prominent cholesterol sulfate band is seen in RXLI than in NHS-SC. Solvent system: tetrohydrofuran, methylal, methanol, ammonium hydroxide (Methods). NL, neutral lipids, unk, unknown lipid, CS, cholesterol sulfate, PL, polar lipids.

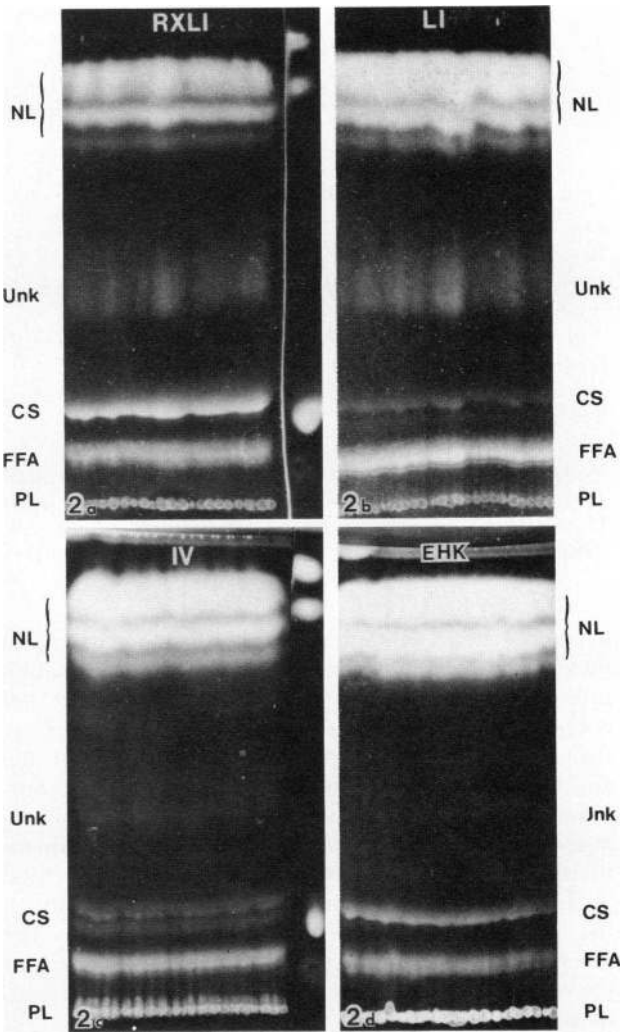


FIGURE 2 Cholesterol sulfate thin layer chromatography of recessive x-linked ichthyotic scale (2a) vs. other ichthyotic scale (2b: Lamellar ichthyosis; 2c: Ichthyosis vulgaris; 2d: Epidermolytic hyperkeratosis). The prominence of the cholesterol sulfate band of RXLI is not seen in other forms of ichthyosis.

(RXLI: 84.3±0.5% vs. NHS-SC: 95.6±0.2%, $P < 0.0025$) (Table V). This difference was due to reduced free sterols (RXLI: 8.9±0.8% vs. NHS-SC: 16.2±2.8%, $P < 0.025$) and increased cholesterol sulfate, since sterol esters, total sterols (combined free sterols, sterol esters, and cholesterol sulfate fractions (Table VI), glycosphingolipids, free fatty acids, triglyceride, and unknown fractions were not significantly different from NHS-SC (data not presented).

## DISCUSSION

*Measurement of stratum corneum cholesterol sulfate.* Because of the limited solubility of cholesterol sulfate in most organic solvents, we and others

TABLE IV
*Distribution of Cholesterol Sulfate in Aqueous and Chloroform Phases during Extraction*

| Disease | Mean percent total lipid±SEM | | |
|---|---|---|---|
| | Chloroform phase | Aqueous phase | Total |
| Recessive x-linked ichthyosis ($n$ = 8) | 10.2±0.8 | 2.6±0.1 | 12.5±0.8* |
| Ichthyosis vulgaris ($n$ = 4) | 1.9±0.7 | 0.8±0.2 | 2.9±0.7‡ |
| Lamellar ichthyosis ($n$ = 4) | 1.0±0.1 | 0.5±0.1 | 1.6±0.2‡ |
| Epidermolytic hyperkeratosis ($n$ = 2) | 1.8 | 0.7 | 2.4‡ |
| Normal ($n$ = 4) | 1.9±0.1 | 1.1±0.6 | 2.6±0.2 |

* $P < 0.0025$.
‡ Not significant.

have observed a tendency for this substance to be lost into the aqueous phase during ordinary lipid extraction procedures (19). As shown in Table IV, the amount of cholesterol sulfate remaining in the aqueous phase using our method of extraction is variable, but can approach 40% of the total cholesterol sulfate. Surmounting the problem of significant cholesterol sulfate loss required our modification of standard quantitative TLC sequences. We have obtained total cholesterol sulfate either by adding the quantity of the cholesterol sulfate-containing gel band to the amount of cholesterol sulfate lost into the aqueous phase, or by extraction of the gel with solvent containing 0.1 M KCl.

In this study, we have shown that RXLI scales contain about five times more cholesterol sulfate than normal outer human stratum corneum (mean, 12.5 vs. 2.6%), or other forms of ichthyosis. Since there is no overlap with normals or other forms of ichthyosis, determination of cholesterol sulfate content of scale offers an alternative method to enzymatic assay for the diagnosis of this disorder. Our current chromatographic methods require only 20 mg of scale for accurate quantitation; future modifications using an Iatroscan (Newman-Howells Assoc., Ltd., Winchester, England) can be expected to reduce this requirement further (20).

*Role of cholesterol sulfate in pathogenesis of RXLI.* These studies show for the first time that the presumed metabolic blockade in RXLI, deficiency of steroid sulfatase, is associated with accumulation of the metabolic precursor, cholesterol sulfate, within the pathologic material, ichthyotic scale. This finding provides strong evidence that absence of the enzyme is directly responsible for the ichthyosis (8). Although it is tempting to speculate that cholesterol sulfate provokes the pathological scaling in RXLI, such a link remains to be established. Moreover, it could be argued that the reduction in free sterols (Table V) might be of more overriding importance than the elevation in cholesterol sulfate. This possibility deserves careful consideration in view of the ichthyotic syndromes that have been described as a side-effect of several cholesterol-lowering drugs (see below).

Very little is known about the distribution of cholesterol sulfate in normal tissues (19), however, this substance has been found in small quantities in plasma, liver, kidney, aorta, adrenal, and brain (19, 21), and in erythrocyte membranes (22). Although its function in these tissues is not known, a role in membrane stabilization has been postulated for sperm (23) and erythrocytes membranes (22). Recently, increased cholesterol sulfate has been detected in the plasma of patients with RXLI where it is carried predominantly in the low density lipoprotein fraction, altering its electrophoretic mobility (24). Since the quantity of cholesterol sulfate in RXLI scale (>1% of total stratum corneum weight) far exceeds the levels detected in RXLI plasma, it is likely that the observed accumulation of cholesterol sulfate in scale is a direct result of epidermal biosynthetic activity, rather than merely a passive reflection of increased plasma choles-

TABLE V
*Cholesterol Sulfate and Other Lipid Fractions from RXLI Scales vs. NHS-SC*

| Disease | Mean percent total lipid±SEM | | Cholesterol sulfate‡ |
|---|---|---|---|
| | Polar lipids | Neutral lipids* | |
| Recessive x-linked ichthyosis ($n$ = 8) | 2.9±0.2 | 84.3±0.5 | 12.5±0.8 |
| Normal ($n$ = 4) | 1.6±0.1 | 95.6±0.8 | 2.6±0.2 |
| | NS | $P < 0.0025$ | $P < 0.0025$ |

* Includes glycosphingolipids.
‡ Sum of cholesterol sulfate in aqueous and chloroform phases.

TABLE VI
*Free Sterols, Sterol Esters and Total Sterols in RXLI Scales vs. NHS-SC*

| Disease | Mean percent total lipid±SEM | | |
|---|---|---|---|
| | Free sterols | Sterol esters | Total sterols* |
| Recessive x-linked ichthyosis ($n$ = 5) | 8.9±0.8 | 4.2±0.8 | 21.6±1.6 |
| Normal ($n$ = 4) | 16.2±2.8 | 5.5±0.5 | 23.4±1.4 |
| | $P < 0.025$ | NS | NS |

* Sum of cholesterol sulfate, free sterols, and sterol esters.

terol sulfate content. On the other hand, serum levels of sulfated steroid hormones do not appear to be altered in RXLI patients (25), and therefore it is less likely that sulfated steroid hormones play a role in the pathogenesis of this disorder.

*Role of stratum corneum lipids in normal and abnormal desquamation.* The factors that control normal, orderly desquamation of outer stratum corneum are as yet poorly understood. It is possible, however, that stratum corneum lipids play an important role in this process (12). Lipids constitute ~10% of normal stratum corneum by weight where they are found predominantly within the intercellular spaces (11) as broad, laminated sheets (12). Thus, by morphological, cytochemical, and biochemical criteria, lipids appear to reside in the correct location to play a potentially prominent role in cohesion and desquamation (15).

Perhaps the strongest evidence for a role of stratum corneum lipids in the modulation of stratum corneum desquamation derives from the observation that a number of scaling disorders occur in acquired or inherited defects of lipid metabolism. Ichthyosis, palmarplantar keratoderma, and acanthosis nigricans, occur as side effects of several drugs (26–29) that interfere with cholesterol biosynthesis or lower serum cholesterol by as yet unknown mechanisms. As reported here, RXLI scales contain not only increased cholesterol sulfate, but also decreased free sterols, raising the possibility that the reduction of free sterols may also regulate desquamation in RXLI. In certain inherited multisystem disorders with ichthyosis, such as multiple sulfatase deficiency (30), neutral lipid storage disease (31), and Refsum's disease (32), a defect in lipid metabolism has been established or strongly suspected. In Refsum's disease, where the ability to oxidize phytanic acid is impaired, epidermal lipids contain large amounts of phytanic acid in the phospholipid, sterol ester, and triglyceride fractions (33). Moreover, a case of Harlequin fetus, a lethal form of congenital ichthyosis, was reported that demonstrated increased quantities of sterols and triglycerides, as well as retained lipid vacuoles, in the stratum corneum (34).

Finally, although their contribution to the overall pathogenesis of these disorders is not established, altered epidermal or systemic lipid metabolism occurs in common scaling disorders, such as psoriasis (35–37), atopic dermatitis (38), lichen simplex chronicus (35), and essential fatty acid deficiency (39).

It remains to be shown how the accumulation of cholesterol sulfate in RXLI scale results in prolonged stratum corneum retention. If cholesterol sulfate, like other stratum corneum lipids, is found to be primarily intercellular in locale, one might speculate that it increases cellular adhesiveness by alterations in net surface charge. Alternatively, increased cholesterol sulfate and/or the observed concommitant decrease in free sterols may affect the phase-transition properties of intercellular lipids (15), thereby causing increased stratum corneum retention.

In summary, the content of cholesterol sulfate is markedly increased in RXLI scale. Since altered lipid metabolism has been implicated in other scaling disorders, it is likely that increased cholesterol sulfate and/or decreased free sterol content are directly related to the pathogenesis of this disorder, although the mechanism involved is as yet unknown. Finally, measurement of cholesterol sulfate content of RXLI scale provides an alternative method for diagnosis.

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