Effects of cholesterol sulfate on lipid metabolism in cultured human keratinocytes and fibroblasts

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Abstract Effects of cholesterol sulfate on acetate incorporation into lipid fractions were examined in normal human fibroblast and keratinocyte cultures. Inhibition of sterologenesis in normal fibroblast cultures by cholesterol sulfate was less profound than that produced by either lipoprotein-containing serum or 25-hydroxycholesterol. Cholesterol sulfate also inhibited sterologenesis in low density lipoprotein receptor-deficient fibroblasts and inhibited both sterologenesis and 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in keratinocytes. Cholesterol sulfate increased incorporation of acetate into fatty acid-containing lipids in preconfluent cultures of both cell types in lipoprotein-depleted media. Similar effects were not observed either in response to lipoprotein-containing serum or 25-hydroxycholesterol. Cholesterol sulfate had no effect on oleic acid incorporation into diglycerides, triglycerides, or phospholipid fractions; neither did it inhibit acid lipase activity; nor did it inhibit fatty acid oxidation, indicating that cholesterol sulfate does not inhibit catabolism of acyl lipids. Because cholesterol sulfate had similar effects on fatty acid metabolism in steroid sulfatase-deficient fibroblasts lines, desulfation to cholesterol is not a prerequisite. Cholesterol sulfate did not significantly affect incorporation of oleic acid into sterol esters in fibroblast cultures, but in contrast, inhibited sterol esterification in keratinocyte cultures. These data suggest a novel role for cholesterol sulfate as a modulator of cellular lipid biosynthesis. — **Williams, M. L., S. L. Rutherford, and K. R. Feingold.** Effects of cholesterol sulfate on lipid metabolism in cultured human keratinocytes and fibroblasts. *J. Lipid Res.* 1987. **28:** 955–967.

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Although cholesterol sulfate is distributed widely in mammalian tissues (1, 2), its function remains uncertain. Postulated roles include a precursor for steroid hormone biosynthesis, a membrane stabilizer, and an excretory product (3–5). Recently, attention has focused on the function of cholesterol sulfate in skin, because deficiency of its hydrolytic enzyme, steroid sulfatase (sterol sulfate sulfohydrolase, EC 3.1.6.2) has been found to underlie the genetic skin disease, recessive X-linked ichthyosis (RXLI) (6). Pathological scale in RXLI contains a fivefold excess of cholesterol sulfate with a corresponding reduction in free sterol content (7); furthermore, normal stratum corneum also contains substantial quantities of cholesterol sulfate (8). Whereas the cholesterol–cholesterol sulfate ratio in blood and gastrointestinal epithelia is approximately 500:1 (9, 10), in normal stratum corneum this ratio is approximately 10:1 and in RXLI, approximately 1:1 (7). Regulation of the cholesterol–cholesterol sulfate ratio in the stratum corneum membrane complexes may be critical to normal desquamation (11).

Recently, following the observation that cholesterol sulfate inhibits cholesterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (mevalonate:NADP[+] oxidoreductase (CoA-acylating), EC 1.1.1.34) activity in both normal and steroid sulfatase-deficient fibroblast cell lines (12), an alternative or additional role for cholesterol sulfate as a modulator of cholesterol metabolism has been proposed. The studies reported here suggest a still broader role for cholesterol sulfate as a modulator of lipid metabolism. In cells of both mesenchymal (i.e., dermal fibroblasts) and epithelial (i.e., foreskin keratinocytes) origin, not only does cholesterol sulfate inhibit sterologenesis, but it also stimulates incorporation of acetate into fatty acid-containing lipids. Evidence is presented that the effect on fatty acid metabolism is not a direct consequence of HMG-CoA reductase inhibition, nor does it require intracellular desulfation. Moreover, the effect is dependent on the cell density of the cultures and on the absence of exogenous lipoproteins in the media. Finally, evidence is presented that the effect is not one of impaired fatty acid catabolism; under certain conditions of culture, cholesterol sulfate appears to stimulate fatty acid synthesis.

Abbreviations: RXLI, recessive X-linked ichthyosis; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLC, thin-layer chromatography; LDL, low density lipoprotein.

MATERIALS AND METHODS

Chemicals

Cholesterol sulfate, sodium salt, was obtained from Research Plus (Bayonne, NJ). The purity of this compound has been confirmed by thin-layer chromatography (12). Mass spectroscopy (performed courtesy of Dr. Joanne Whitney, U.C.S.F. Mass Spectrometry Unit) demonstrated less than 0.1% contamination with probable oxygenated (sulfated) sterol by-products. 25-Hydroxycholesterol was obtained from Steraloids (Wilton, NH). [^{3}H]Cholesterol sulfate was synthesized from [1,2,6,7-^{3}H(n)]cholesterol (sp act 82.7 Ci/mmol; New England Nuclear; Boston, MA) as previously described (12). [9,10,(n)-^{3}H]Oleic acid (sp act 4.8 Ci/mmol) was obtained from Amersham, and conjugated to fatty acid-free bovine serum albumin (Sigma; St. Louis, MO).

Fibroblast cell culture

Normal human fibroblast lines were obtained from neonatal foreskins, and maintained on DME-H21 media (Gibco Laboratories; Grand Island, NY) supplemented with 10% newborn calf serum. Both RXLI lines were derived from patients who demonstrated an absence of steroid sulfatase activity in leukocyte preparations (13) (enzyme assays performed courtesy of Dr. Ervin H. Epstein, Jr.). Desulfation of cholesterol sulfate by RXLI lines was less than 6% that of normal (12). Low density lipoprotein (LDL) receptor-deficient fibroblast cell line (GM 701) was obtained from NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). All studies were done on low-passage lines (< 15), and in triplicate. Cell culture experiments were carried out on 100 × 15 mm Petri dishes in 10 ml of media at an initial plating density of approximately 10^5 cells per dish.

Keratinocyte cell culture

Primary cultures of human keratinocytes were prepared from trypsin- and collagenase-treated human neonatal foreskins by a modification of the method of Rheinwald and Green (14). Cells were passaged onto 100-mm tissue culture dishes at a plating density of approximately 10^6 cells per dish, previously seeded with a mitomycin C-treated 3T3 mouse fibroblast feeder layer. Feeder cells were selectively removed by washing with 0.01% EDTA in calcium, magnesium-free Dulbecco's buffer (Gibco Laboratories) 2 or 3 days prior to each experiment. Keratinocyte cultures were grown on Dulbecco's modified Eagle's media (DMEM, 4.5 g of glucose/liter; Gibco Laboratories) supplemented with 10% fetal calf serum (Whittaker M.A. Bioproducts, Walkersville, MD), 0.4 μg/ml hydrocortisone (Sigma; St. Louis, MO), 0.1 μg/ml cholera toxin (Sigma), 20 ng/ml mouse submaxillary gland epidermal growth factor (Collaborative Research; Lexington, MA), 2.5 μg/ml Fungizone (Gibco Laboratories), and 1.0 μg/ml glutamine (Gibco Laboratories). Cells were routinely washed with Dulbecco's phosphate-buffered saline (PBS) (Gibco Laboratories) and given fresh media three times a week. All experiments were performed with first-to-third passage lines.

Acetate incorporation into lipid

Sera were depleted of lipoprotein by potassium bromide-density gradient centrifugation (15) and dialyzed to remove added salts. Cholesterol sulfate and 25-hydroxycholesterol were dissolved in methanol and added to the media (final concentration of methanol < 0.25%); controls for each experiment received an equivalent amount of methanol. DL-α-Tocopherol (Sigma) (1 μg/ml) was solubilized in ethanol (final concentration of ethanol 0.05%). Cells were exposed to media containing serum or lipoprotein-depleted serum, sterols, vehicles for 24 hr; lipid synthesis was determined in fibroblasts by incubation with 1 μCi/ml [1-^{14}C]acetate (sp act 57.5 Ci/mol; New England Nuclear) or [1-^{3}H]sodium acetate (sp act 1.6 μCi/nmol; New England Nuclear) in fresh media with additives for an additional 12–24 hr prior to harvesting. Keratinocyte cultures were given additional unlabeled acetate (5 μM; Sigma). Heat-killed cultures do not incorporate significant quantities of radiolabeled acetate into lipid. Our method for harvesting and lipid extraction has been described previously (12). Cellular lipid extracts were analyzed by thin-layer chromatography (TLC) on silica-gel plates (Merck, Darmstadt) in a two-step solvent system. Plates were developed first to 14 cm in benzene–diethyl ether–ethanol–acetic acid 50:40:2:0.2 (by vol), dried, and then developed in petroleum ether–diethyl ether–glacial acetic acid 80:20:1 (by vol). This system permits separation of diglycerides from free sterols. However, in some runs diglycerides merged with free fatty acids. Plates were lightly sprayed with 0.25% 8-anilino-1-naphthalene in water, and lipid bands were visualized under black-light fluorescence. TLC fractions were placed in Scintiverse II (Fisher Scientific; Springfield, NJ), and the radioactivity in each fraction was counted in a Beckman LS-1800 scintillation counter. In other experiments, TLC plates were scanned with a Berthold LB-283 TLC linear analyzer.

For determination of acetate incorporation into saponifiable lipids, cell pellets were incubated at room temperature overnight in 45% potassium hydroxide–water–ethanol 2:1:5 (by vol). Nonsaponifiable lipids were obtained by extraction three times with petroleum ether. Saponifiable lipids were obtained by acidification to pH 2.0, followed by extraction three times with petroleum ether.

Carbon dioxide production

Fibroblasts in 25-cm^2 flasks (Corning Glass Works) were given 10% newborn calf serum or 10% lipoprotein-

depleted newborn calf serum, plus cholesterol sulfate, 25-hydroxycholesterol, or vehicle alone (0.25% methanol; control) 12 to 24 hr prior to assay. [^{14}C]Carbon dioxide production from [1-^{14}C]palmitic acid (5.7 mCi/mmol; New England Nuclear) or from [1-^{14}C]acetate was assayed by a modification of the method of Rodbell (16). Briefly, media were decanted, and cultures were placed in Dulbecco's phosphate-buffered saline (Gibco Laboratories) with 0.5 μCi/ml of labeled compound. Palmitic acid was previously conjugated to fatty acid-free bovine serum albumin (Sigma). Flasks were sealed with rubber-sleeve stoppers that were fitted with a collection well containing a strip of Whatman #1 filter paper. After incubation at 37°C for 1 hr, the reaction was stopped by the addition of sulfuric acid (final concentration, 0.1 N). Hyamine hydroxide (0.2 ml; ICN; Irvine, CA) was added to the wells, and carbon dioxide was collected for 1 hr. Filter paper strips were placed in vials with scintillation fluid (ACS; Amersham) and immediately counted in a Beckman LS-1800 scintillation counter. To minimize interference due to chemiluminescence, samples were counted with a window that excluded the lower 25% of the spectrum. Cells were harvested with a rubber policeman, and assayed for protein (17) and DNA (18) content.

HMG-CoA reductase activity

Keratinocytes were washed three times with 20 mM imidazole, 5 mM dithiothreitol buffer (pH 7.4), and centrifuged for 2 min at 1000 rpm. The pellet was resuspended in buffer and homogenized in a ground-glass homogenizer to provide a suspension of broken cells. HMG-CoA reductase activity was assessed as the conversion of [3-^{14}C]HMG-CoA to mevalonate, as previously described (19).

Lipase assay

Lipase assay was performed by modification of methods of Severson (20) and Knauer and Weglicki (21). Cultures were rinsed with phosphate-buffered saline and harvested by scraping dishes with a rubber policeman. Cells were homogenized by repeated, forceful aspiration through a syringe fitted with a 30-gauge needle. The substrate for this assay was tri[1-^{14}C]oleoylglycerol (111.8 mCi/mM; New England Nuclear) previously purified of contaminants by thin-layer chromatography in petroleum ether–diethyl ether–acetic acid 80:20:1 (by vol). To this was added a 4-fold excess of triolein (Sigma) in hexane and a 25-fold quantity by weight of egg phosphatidylcholine (Sigma) in chloroform. The lipid mixture was dried under nitrogen, suspended in 500 μl of phosphate-buffered saline, and sonicated for 15 min at 40°C. Lipase activity was assayed at pH 4.5. An incubation mixture consisting of 670 μl of 50 mM sodium acetate plus 50 μl of substrate (2×10^5 cpm) was vigorously vortexed and the assay was initiated by the addition of 300 μl of cellular homogenate followed by vigorous vortexing. All incubations were performed in duplicate at 37°C for 1 hr. The reaction was stopped by the addition of chloroform (6 vol), acidified to pH 2, and the mixtures were vigorously shaken on a Burrell® wrist-action shaker for 10 min, and centrifuged at 2000 *g* for 10 min. The upper, aqueous phase was discarded, and the organic phase was dried under nitrogen, resuspended in chloroform, and chromatographed in the solvent system outlined above. Free fatty acid and triglyceride regions were identified by cochromatography of unlabeled standard and visualization under black-light fluorescence after spraying with 0.25% 8-anilino-1-naphthalene. The triglyceride and free fatty acid regions of the chromatograms were transferred directly into vials containing 10 ml Scintiverse II (Fisher) for determination of radioactivity by liquid scintillation spectrometry, with external standardization to determine counting efficiency.

RESULTS

Effect of cholesterol sulfate on sterologenesis

Cholesterol sulfate at 10 μM concentrations in lipoprotein-depleted media inhibited acetate incorporation into cholesterol by over 50% (**Fig. 1**). Both lipoprotein-containing serum (10% serum) and 25-hydroxycholesterol (1.25 μM) were significantly more inhibitory to sterologenesis than was cholesterol sulfate, achieving maximal inhibition of approximately 90% and 95%, respectively, in parallel cultures (Fig. 1). While the percent inhibition of acetate incorporation into free sterols varied substantially from experiment to experiment due to marked differences in sterologenesis rates depending upon the density and growth characteristics of the cultures (12, 22, 23), within an experiment, cholesterol sulfate always inhibited sterol synthesis in normal fibroblasts to a lesser extent than did either lipoprotein-containing serum or 25-hydroxycholesterol.

While previous studies had shown that TLC-purified and stock cholesterol sulfate were equally inhibitory to sterologenesis (12), the possibility that auto-oxygenated by-products of cholesterol sulfate formed during cell culture incubations and that these by-products were the inhibitors of sterologenesis was not examined. Therefore, we examined the ability of cholesterol sulfate to inhibit sterologenesis when incubated in the presence of the antioxidant, α-tocopherol (**Table 1**). While the antioxidant alone had no effect on sterologenesis, marked inhibition was observed with antioxidant plus cholesterol sulfate. Furthermore, this inhibition significantly exceeded that produced by cholesterol sulfate alone, suggesting the possibility that an oxygenated derivative may indeed be formed during prolonged incubations, but is inactive as an inhibitor of sterologenesis. Alternatively, α-tocopherol may increase the effectiveness of cholesterol sulfate by

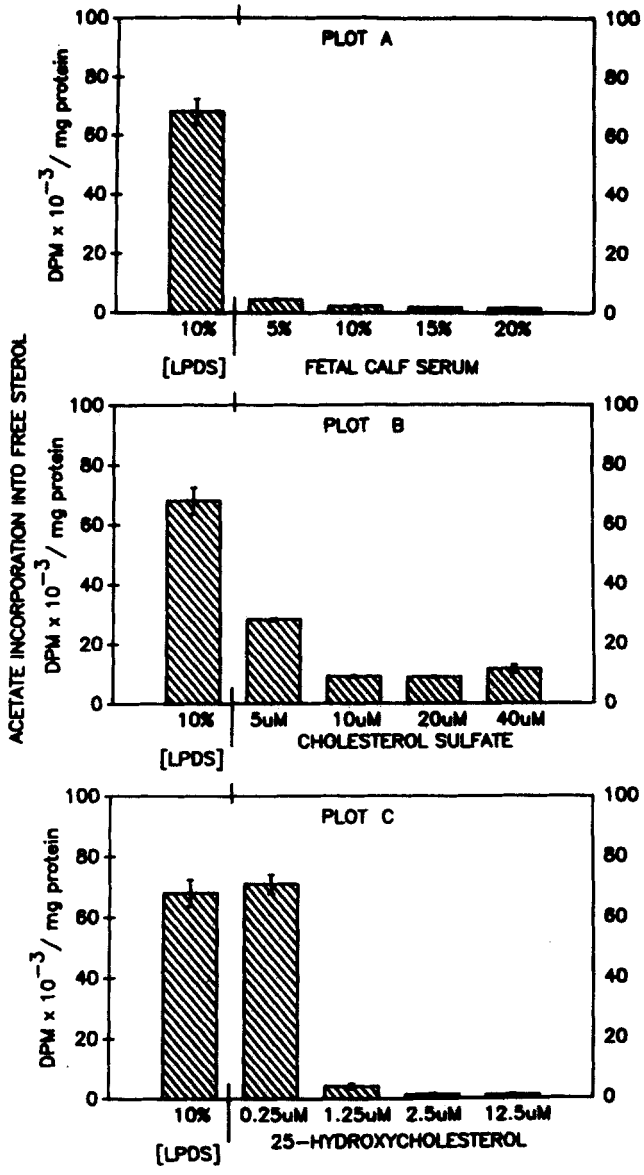

Fig. 1. Comparison of effect of cholesterol sulfate, serum containing lipoproteins and 25-hydroxycholesterol on sterologenesis in fibroblasts. Forty-eight hr prior to harvesting, preconfluent fibroblast cultures were placed in 10% lipoprotein-depleted (LPDS) or 5, 10, 15, and 20% lipoprotein-containing newborn calf serum plus vehicle (0.25% methanol; panel A). Parallel cultures were similarly pulsed with cholesterol sulfate (panel B) or 25-hydroxycholesterol (panel C) in 0.25% methanol and 10% lipoprotein-depleted serum. Twenty-four hr prior to harvesting, media and additives were renewed and [^{14}C]acetate was added. Data are expressed as incorporation of acetate into free sterols, dpm x 10^{-3} per mg protein, a mean of three dishes each ± SEM.

some other mechanism, such as by increasing cellular transport or by inhibiting desulfation.

Because cholesterol sulfate is normally concentrated in epidermis, we examined the effects of cholesterol sulfate on sterologenesis in foreskin keratinocytes. Unlike fibroblasts in culture, keratinocyte growth is both horizontal and vertical, so that confluent cultures are multilayered and exhibit many features of differentiation similar to epidermis in vivo (24), and may remain stable for several weeks without passage. Ponec et al. (25) have demonstrated that confluent keratinocyte cultures do not express low density lipoprotein (LDL) receptors and do not modulate sterologenesis in response to exogenous lipoproteins in the media. However, sterologenesis in preconfluent keratinocyte cultures is decreased when lipid-containing sera are added to the media (26), suggesting that LDL receptors are probably expressed at this stage in culture. Hence, we examined the effect of cholesterol sulfate on acetate incorporation into free sterols in both preconfluent and confluent keratinocyte cultures (**Fig. 2**). In both states of confluency, 10 μM cholesterol sulfate significantly inhibited incorporation of acetate into free sterols when keratinocytes were in lipoprotein-depleted media (Fig. 2). In preconfluent cultures, sterologenesis was inhibited by 10% fetal calf serum and there was no further inhibition by 10 μM cholesterol sulfate (Fig. 2, left panel). In contrast, confluent cultures demonstrated no significant modulation of sterologenesis in response to lipoprotein-containing versus lipoprotein-depleted serum (Fig. 2, right panel), in agreement with the observations of Ponec et al. (25). However, 50 μM cholesterol sulfate was inhibitory under both conditions of culture, and it was significantly more inhibitory to cultures in lipoprotein-depleted media.

Cholesterol sulfate uptake by both keratinocytes and fibroblasts is not a receptor-mediated process (27). Cholesterol sulfate, which is carried primarily by LDL in serum (28), would not be available to cells lacking LDL receptors, i.e., confluent keratinocytes, if the cholesterol sulfate partitioned into LDL when placed in lipoprotein-containing media. In order to examine this possibility, we added 10 and 50 μM cholesterol sulfate (6 x 10^5 dpm) to media containing 10% fetal calf serum, and incubated the media for 48 hr at 33°C, then separated the lipoprotein fraction by density gradint centrifugation and determined cholesterol sulfate uptake. Under these conditions, approximately 85% of the label partitioned into the lipoprotein fraction. If all cholesterol sulfate in lipoprotein-depleted media were available to confluent keratinocyte cultures, but only 15% of cholesterol sulfate were available to the cells in the presence of 10% lipoprotein-containing serum, then 50 μM cholesterol sulfate added to lipoprotein-containing media would represent the equivalent of 7.5 μM in lipoprotein-depleted media. This prediction corresponds well to observed acetate incorporation into sterols in confluent keratinocytes by 10 μM cholesterol sulfate in lipoprotein-depleted media versus 50 μM in lipoprotein-containing media (Fig. 2; right panel).

Similarly, cholesterol sulfate inhibited sterologenesis in the presence of either lipoprotein-containing or lipoprotein-depleted serum in fibroblasts derived from a homozygote for LDL receptor deficiency, but 10 μM cholesterol sulfate produced greater inhibition in these

TABLE 1. Effect of cholesterol sulfate on incorporation of acetate into free sterols in the presence or absence of the antioxidant, α-tocopherol

Condition	Acetate to Free Sterol	% Control Significance
	dpm × 10^{-3}/μg *DNA*	
LPDS	514.9 ± 19.6	
LPDS + α-tocopherol	548.9 ± 69.6	107%, NS
LPDS + CS	328.2 ± 39.5	64%, $P < 0.02$
LPDS + α-tocopherol + CS	165.3 ± 20.2	30%, $P < 0.01$

Preconfluent fibroblast cultures were exposed to 10 μM cholesterol sulfate (CS), 1 μg/ml α-tocopherol, both, or vehicle alone (0.25% methanol), in 10% lipoprotein-depleted media (LPDS) for 24 hr, then given fresh media plus additives and labeled with [^{3}H]acetate (5 μCi/dish; sp act 1.6 Ci/mmol) for 24 hr. Cultures were harvested, lipids were extracted, and the incorporation of acetate into free sterols was determined. Data represent means of three dishes each ± SEM. Significance was determined by Student's *t*-test; NS, not significant.

cells in the presence of lipoprotein-depleted media (**Table 2**). Again, comparable levels of inhibition could be achieved in lipoprotein-containing media by higher concentrations of cholesterol sulfate (e.g., 50 μM). These differences can be attributed to partition of cholesterol sulfate to LDL, a fraction that would be less available to cells not expressing LDL receptors. An unanticipated finding was decreased free sterol synthesis in these cells in lipoprotein-depleted versus lipoprotein-containing media. We also observed a similar reduction in acetate incorporation into total lipids and into phospholipids (data not shown), thus suggesting removal of a factor necessary for optimal cell growth during preparation of lipoprotein-deficient serum.

It has been previously shown in whole cell preparations of fibroblasts that cholesterol sulfate treatment results in inhibition of HMG-CoA reductase activity (12). In lipoprotein-depleted media, 1.25 μM 25-hydroxycholesterol and 10 μM cholesterol sulfate inhibited HMG-CoA reductase activity in preconfluent keratinocytes by 84 and 68%, respectively.

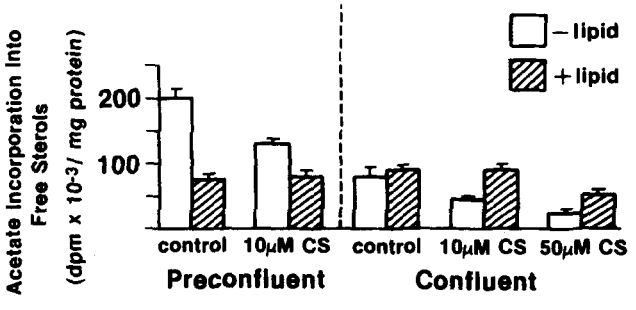

Fig. 2. Effect of cholesterol sulfate on sterologenesis in keratinocytes. Forty-eight hr prior to harvesting, preconfluent (left panel) or confluent (right panel) keratinocyte cultures were placed in 10% lipoprotein-containing (+ lipid; closed bars) or lipoprotein-depleted (– lipid; open bars) fetal calf serum, with and without cholesterol sulfate (CS) (10 μM in preconfluent; 10 or 50 μM in confluent cultures) and 0.25% methanol (see legend to Fig. 1).

Effect of cholesterol sulfate on incorporation of acetate into fatty acid-containing lipids

In contrast to its inhibitory effect on synthesis of free sterols, cholesterol sulfate increased acetate incorporation into phospholipids, free fatty acids/diglycerides, and triglycerides in preconfluent fibroblast cultures (panel B, **Fig. 3, Fig. 4, Fig. 5**). The triglyceride fraction appeared to be most sensitive to this effect, with increased acetate incorporation produced by 10 μM cholesterol sulfate (panel B, Fig. 5). Stimulation of acetate incorporation into free fatty acid/diglycerides and phospholipids was evident with cholesterol sulfate at 25 μM or greater concentrations (panel B, Figs. 3, 4).

To compare the effects of cholesterol sulfate with those of serum-containing lipids on acetate incorporation into these lipid classes, parallel fibroblast cultures were treated with either 10% lipoprotein-depleted serum or lipoprotein-containing serum at 5, 10, 15, or 20% (panel A, Figs. 3–5). Lipoprotein-containing serum significantly inhibited acetate incorporation into phospholipids and free fatty acids/diglycerides (panel A, Figs. 3, 4). These results are consistent with prior work demonstrating the dependence of de novo lipid synthesis in cultured fibroblasts on the availability of exogenous lipid (23). The possibility existed

TABLE 2. Effect of cholesterol sulfate on sterol synthesis in LDL receptor-deficient fibroblasts

Media	Acetate Incorporation into Free Sterol	% Control
10% Serum + vehicle	102.4 ± 29.1	
10% Serum + 10 μM CS	40.5 ± 1.3	40
10% Serum + 50 μM CS	3.2 ± 0.5	3
10% LPDS + vehicle	66.3 (n = 2)	
10% LPDS + 10 μM CS	15.7 ± 2.0	24

Preconfluent cultures of LDL receptor-deficient fibroblasts (GM 701, see Methods) were placed in 10% newborn calf serum or in 10% lipoprotein-depleted serum (LPDS) plus cholesterol sulfate in 0.5% methanol or vehicle alone for 24 hr, then placed in fresh media plus additives and labeled with [^{3}H]acetate (5 μCi/dish; sp act 1.6 Ci/mmol) for 12 hr. Data are expressed as acetate incorporation into free sterol, dpm + 10^{-3}/mg protein, a mean of three dishes each ± SEM.

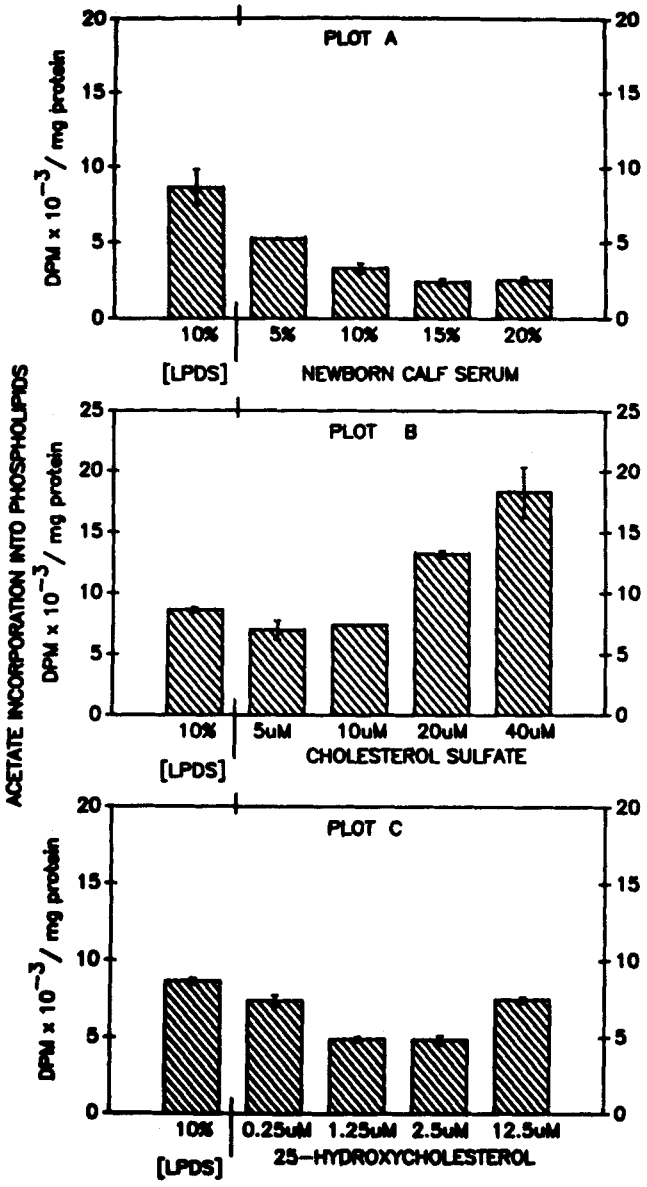

Fig. 3. Phospholipid synthesis: comparison of effects of serum lipids, cholesterol sulfate and 25-hydroxycholesterol. See legend to Fig. 1. Panel A: serum; panel B: cholesterol sulfate; panel C: 25-hydroxycholesterol. Data are expressed as acetate incorporation into phospholipids, dpm × 10^{-3} per mg of protein, mean of three dishes ± SEM.

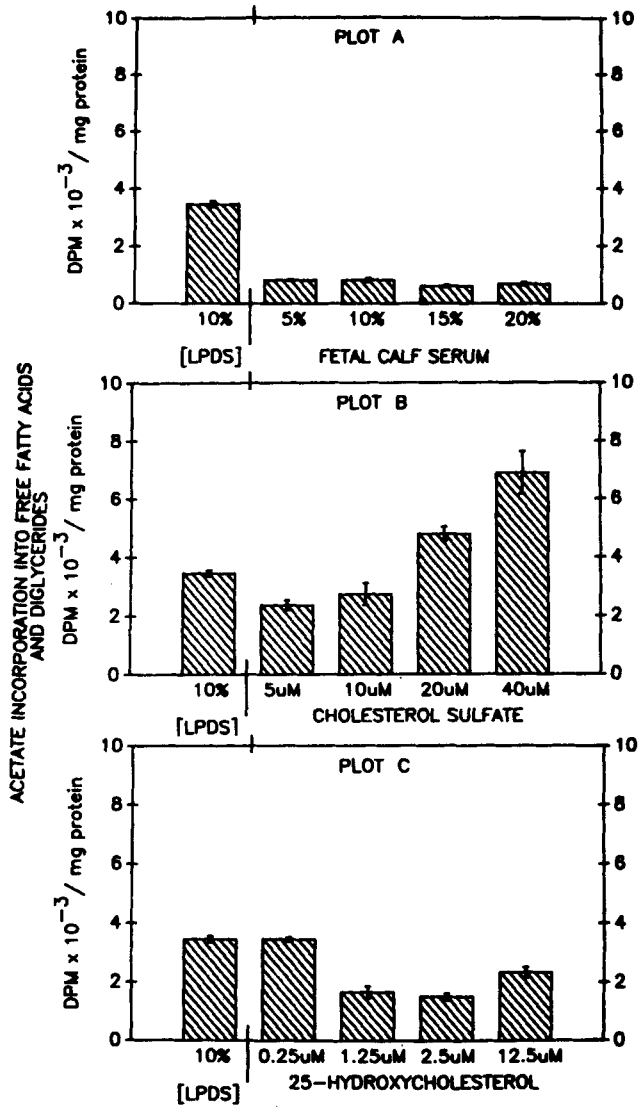

Fig. 4. Free fatty acid/diglyceride synthesis: comparison of effects of serum lipids, cholesterol sulfate and 25-hydroxycholesterol. See legend to Fig. 1. Panel A: serum; panel B: cholesterol sulfate; panel C: 25-hydroxycholesterol. Data are expressed as acetate incorporation into free fatty acids and diglycerides, dpm × 10^{-3} per mg of protein, mean of three dishes ± SEM.

that the increased incorporation of acetate into nonsterol lipids in the presence of cholesterol sulfate was due to shunting of available acetate from an inhibited pathway to a noninhibited pathway (i.e., from sterologenesis to fatty acid synthesis), whereas lipoprotein-containing serum would inhibit both pathways. We therefore examined the effects of acetate incorporation into these lipid fractions in response to 25-hydroxycholesterol, a known potent inhibitor of cholesterol biosynthesis (panel C, Figs. 3–5). While 25-hydroxycholesterol at 1.25 μM and greater concentrations markedly inhibited sterologenesis (panel C, Fig. 1), increased incorporation of acetate into other lipid fractions was not observed (panel C, Figs. 3–5). In other experiments we also examined the effect of 25-hydroxycholesterol in higher concentrations, comparable to those used with cholesterol sulfate; no increased incorporation into fatty acid-containing fractions was observed at these higher concentrations either (data not shown). However, some concentrations of 25-hydroxycholesterol were inhibitory to acetate incorporation into phospholipids and free fatty acids/diglycerides (panel C, Figs. 3–5). Finally, the dissociation of dose-response curves for incorporation of acetate into sterols versus other lipid fractions in cholesterol sulfate-treated cells (panel B, Figs. 1 and 3–5),

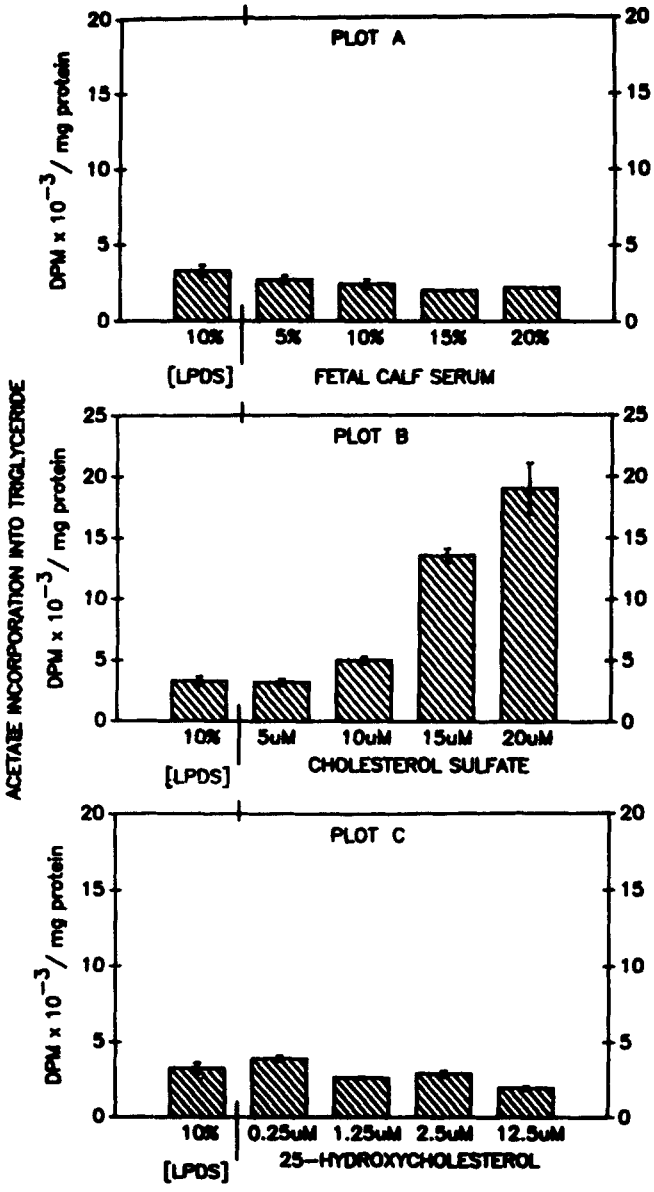

Fig. 5. Triglyceride synthesis: comparison of effects of serum lipids, cholesterol sulfate and 25-hydroxycholesterol. See legend to Fig. 1. Panel A: serum; panel B: cholesterol sulfate; panel C: 25-hydroxycholesterol. Data expressed as dpm/mg of protein, mean of three dishes ± SEM.

suggests that the increased incorporation into nonsterol lipids cannot be ascribed to shifting substrate pools.

To determine whether exogenous lipoproteins modulated the effect of cholesterol sulfate on incorporation of acetate into fatty acid-containing lipid fractions, preconfluent fibroblasts were cultured for 48 hr in either 10% lipoprotein-depleted or lipoprotein-containing serum with cholesterol sulfate (10 or 40 μM) or vehicle alone (**Fig. 6**). A stimulatory effect of cholesterol sulfate on acetate incorporation into fatty acids/diglycerides and triglycerides was observed only in lipoprotein-depleted media (Fig. 6). 25-Hydroxycholesterol did not affect acetate incorporation into these lipid fractions in either media (Fig. 6).

To determine whether differences in cell density modulated the cholesterol sulfate effect on acetate incorporation into fatty acid-containing lipids, early (1 day), preconfluent (3 day), and confluent (5 day) fibroblast cultures were placed in 10% lipoprotein-depleted serum containing cholesterol sulfate or vehicle alone, and were harvested at 3, 5, and 7 days, respectively. In both early and preconfluent cultures, incorporation of acetate into nonsterol lipids was markedly stimulated in cholesterol sulfate-treated cultures (**Fig. 7**). Although acetate incorporation into triglyceride was significantly increased by cholesterol sulfate treatment in confluent cultures, these changes were modest in comparison to the effect in early and preconfluent cultures (panel D, Fig. 7). These data demonstrate that the impact of cholesterol sulfate on acetate incorporation into fatty acid-containing lipids in fibroblasts, like sterologenesis, is density-dependent, and that these effects are most marked in cultures examined during their logarithmic growth phase.

Similarly, with keratinocyte cultures, the effect of cholesterol sulfate on acetate incorporation into fatty acid-containing lipid fractions was dependent both upon the state of confluence, and also upon the presence or absence of exogenous lipoproteins (**Fig. 8**). In preconfluent, lipoprotein-depleted cultures, cholesterol sulfate significantly increased acetate incorporation into phospholipid (Fig. 8A, left panel), free fatty acids (Fig. 8B, left panel), and triglycerides (Fig. 8D, left panel). These effects were abolished when preconfluent cultures were grown in lipoprotein-containing media. In contrast, in confluent cultures in lipoprotein-depleted media, cholesterol sulfate inhibited acetate incorporation into free fatty acids (Fig. 8B, right panel) and triglycerides (Fig. 8D, right panel).

To determine whether or not the effect of cholesterol sulfate on acetate incorporation into fatty acid-containing lipids required desulfation to cholesterol, we utilized two lines of steroid sulfatase-deficient fibroblasts derived from patients with recessive X-linked ichthyosis. As with normal fibroblasts, steroid sulfatase-deficient fibroblasts exhibited a dose-dependent increase in acetate incorporation into fatty acid-containing lipids in response to cholesterol sulfate (data not shown). These studies demonstrated that, like the effect on sterologenesis (12), prior desulfation to cholesterol is not a prerequisite for the cholesterol sulfate effect on fatty acid metabolism.

We also observed increased acetate incorporation into triglycerides in response to 10 μM cholesterol sulfate in the presence of antioxidant (α-tocopherol) (control: 132 ± 36; α-tocopherol alone: 117 ± 12 (not significant, NS); cholesterol sulfate alone: 263 ± 47 (NS); cholesterol sulfate plus α-tocopherol: 330 ± 21 ($P < 0.01$); dpm/μg DNA; see Methods, legend Table 1). These data indicate

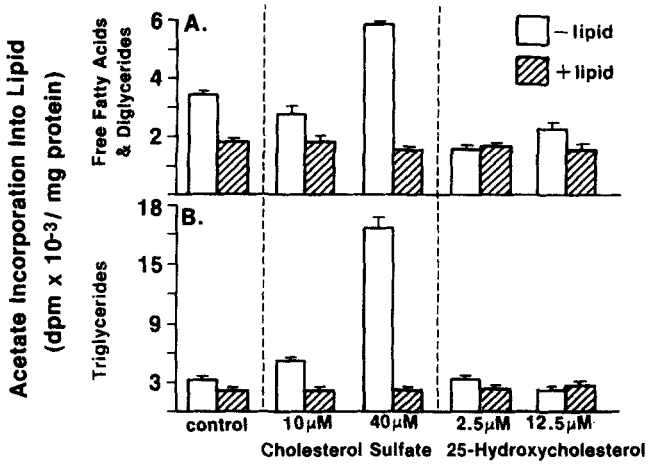

Fig. 6. Dependence of cholesterol sulfate effect on synthesis of fatty acid-containing lipids upon presence or absence of serum lipoproteins in fibroblasts. Incorporation of acetate into free fatty acids/diglycerides (A) and triglycerides (B) was assayed (see legends to Figs. 1 and 3). Parallel preconfluent fibroblast cultures were placed either in 10% lipoprotein-containing (+ lipid; closed bars) or lipoprotein-depleted (− lipid; open bars) sera with vehicle alone (0.25% methanol; left panel), 10 μM and 40 μM cholesterol sulfate (middle panel), or 25 μM and 12.5 μM 25-hydroxycholesterol (left panel).

that the effect on acyl lipids can be attributed to cholesterol sulfate per se rather than to an oxygenated contaminant. Again, the effect observed with cholesterol sulfate and antioxidant exceeds that observed with cholesterol sulfate alone. The failure for 10 μM cholesterol sulfate alone to significantly increase incorporation into triglycerides is not surprising at this concentration (panel B, Fig. 5). The enhanced effect in the presence of antioxidant suggests that α-tocopherol may prevent degradation of cholesterol sulfate, either by preventing generation of an inactive oxygenated metabolite or perhaps by preventing desulfation to cholesterol.

Cholesterol sulfate effects on fatty acid catabolism

Several experiments were undertaken to determine whether the accumulation of radiolabel in fatty acid-containing lipids in cholesterol sulfate-treated cultures was due to impaired catabolism of fatty acids. First, preconfluent fibroblast cultures were grown for 48 hr in 10% lipoprotein-depleted serum in the presence of 10 μM cholesterol sulfate, 1.3 μM 25-hydroxycholesterol or vehicle alone, and pulsed with [^{3}H]oleic acid for the final 24 hr (**Fig. 9**). Cholesterol sulfate did not increase the incorporation of this exogenously supplied fatty acid into any lipid fraction (Fig. 9). Next, we assayed lipase activity in fibroblast cultures pulsed with 10 μM and 20 μM cholesterol sulfate or vehicle alone both in the presence and absence of lipoprotein-containing media. Again, no significant effect of cholesterol sulfate on lipase activity was observed (data not shown).

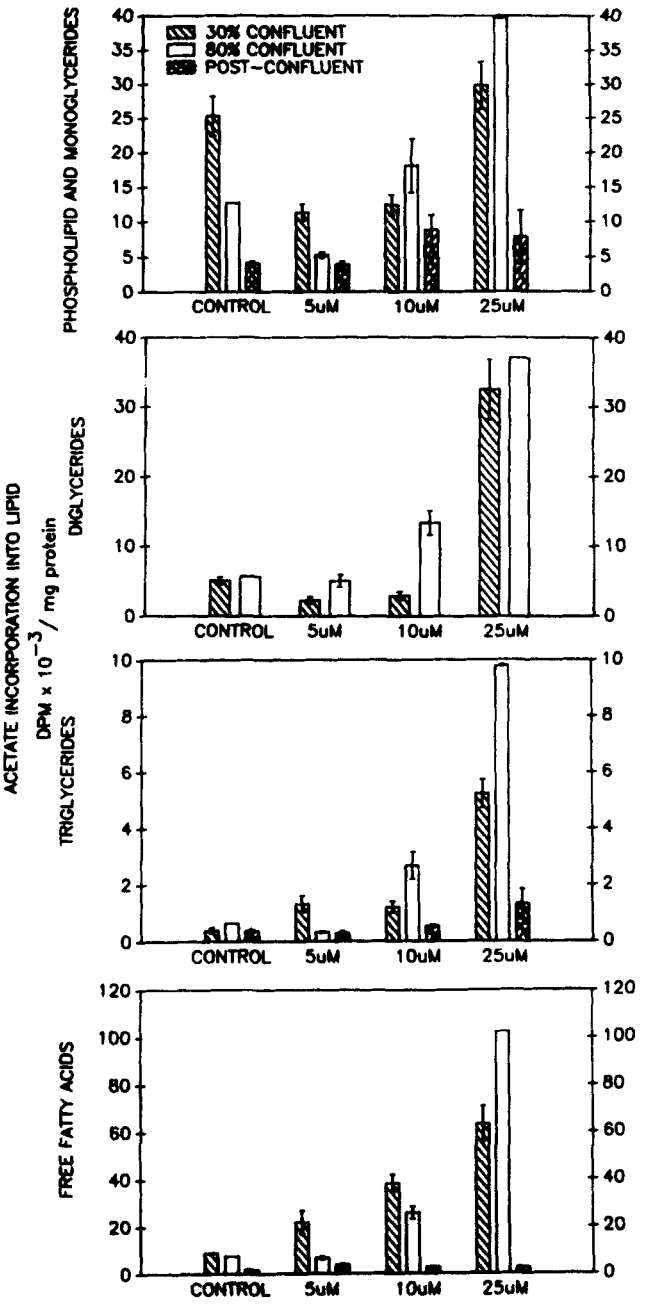

Fig. 7. Density dependence of cholesterol sulfate effect on synthesis of fatty acid-containing lipids in fibroblasts. Parallel fibroblast cultures were seeded on day 1 (seeding density (10^5 cells/100-mm dish)), and harvested at approximately 30% confluence (3 days; single-hatched bars), 80% confluence (5 days; open bars) and postconfluence (10 days; double-hatched bars). In each group, triplicate dishes were placed in 10% lipoprotein-depleted serum plus 5, 10, or 25 μM cholesterol sulfate or vehicle alone (0.25% methanol) 48 hr prior to harvesting. Twenty-four hr prior to harvesting, media and additives were renewed and [^{14}C]acetate was added. Data are expressed as incorporation of acetate into phospholipids (panel A), free fatty acids (panel B), diglycerides (panel C), and triglycerides (panel D), dpm × 10^{-3} per mg of protein, a mean of three dishes each ± SEM.

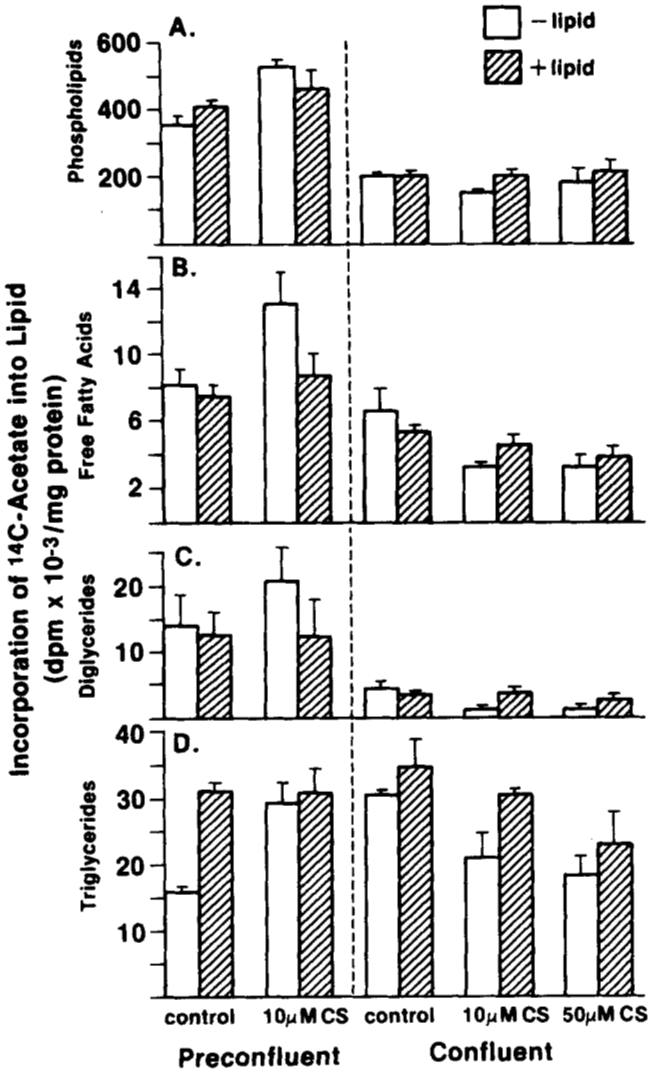

Fig. 8. Dependence of cholesterol sulfate effect on synthesis of fatty acid-containing lipids upon cell density and serum lipoproteins in keratinocyte cultures. Preconfluent (right panel) and confluent (left panel) keratinocyte cultures were placed in 10% lipoprotein-containing (+ lipid; closed bars) or lipoprotein-depleted (– lipid; open bars) sera, with cholesterol sulfate (CS) and/or vehicle for 48 hr, and the incorporation of acetate into phospholipids (A), free fatty acids (B), diglycerides (C), and triglycerides (D) was assayed during the final 24 hr (see legends, Figs. 1 and 2).

Finally, we assayed carbon dioxide production from [1-^{14}C]palmitic acid, as a measure of fatty acid oxidation in preconfluent fibroblast cultures (**Table 3**). No inhibition by cholesterol sulfate was demonstrated. Likewise, cholesterol sulfate had no effect on CO_2 production in cultures grown in lipoprotein-containing media (data not shown). Cholesterol sulfate was only slightly inhibitory to carbon dioxide production from [^{14}C]acetate (20 μM cholesterol sulfate, 92% control; Table 3). Taken together, these experiments demonstrate that the increased incorporation of acetate into fatty acid-containing lipids in response to cholesterol sulfate under certain conditions of cell culture cannot be attributed to impaired triglyceride and fatty acid catabolism, nor can it be attributed to effects on intracellular acetate pools.

Effect of cholesterol sulfate on sterol esterification

25-Hydroxycholesterol and several other inhibitors of HMG-CoA reductase activity also stimulate sterol esterification. This is a direct effect on acyltransferase activity independent of their effect on reductase activity (29). To examine the effect of cholesterol sulfate on sterol esterification, we assayed the incorporation of [^{3}H]oleic acid rather than acetate into sterol esters, since the latter would reflect rates of both sterol and fatty acid synthesis, as well as sterol esterification. Whereas no significant effect of cholesterol sulfate on incorporation of [^{3}H]oleic acid into sterol esters was observed in fibroblasts, in parallel cultures, 25-hydroxycholesterol significantly stimulated esterification of [^{3}H]oleic acid (Fig. 9D). In contrast, cholesterol sulfate did inhibit incorporation of [^{3}H]oleic acid into sterol esters in keratinocyte cultures (**Table 4**); this effect was most pronounced in confluent cultures. As with fibroblasts, sterol esterification in keratinocytes was stimulated by 25-hydroxycholesterol (Table 4).

DISCUSSION

The polar sterol metabolite, cholesterol sulfate, inhibits sterol synthesis and HMG-CoA reductase activity in cul-

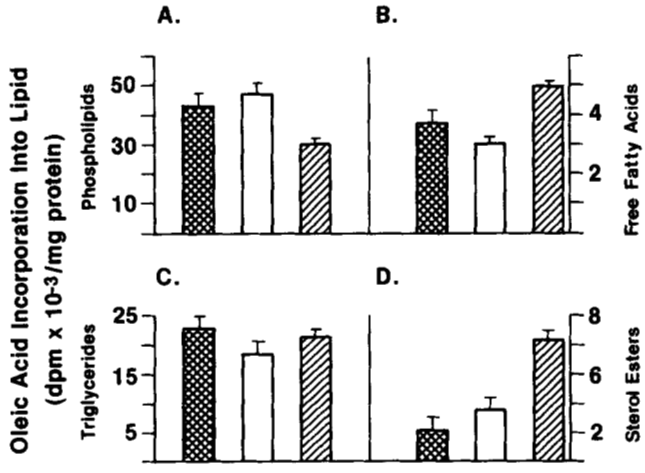

Fig. 9. Effect of cholesterol sulfate and 25-hydroxycholesterol on incorporation of [^{3}H]oleic acid into lipid in fibroblasts. Parallel preconfluent fibroblast cultures were placed in 10% lipoprotein-depleted media and vehicle alone (0.25% methanol; double-hatched bar), 10 μM cholesterol sulfate (open bar) or 1.3 μM 25-hydroxycholesterol (single-hatched bar) 48 hr prior to harvesting. Media and additives were renewed and cultures were incubated with [^{3}H]oleic acid for the final 24 hr. Data are expressed as incorporation of oleic acid into phospholipids (A), free fatty acids (B), triglycerides (C) and sterol esters (D), dpm × 10^{-3} per mg of protein, a mean of three dishes each ± SEM.

TABLE 3. Effect of cholesterol sulfate on [^{14}C]CO_2 production from [^{14}C]palmitate and [^{14}C]acetate in fibroblasts

Media	[^{14}C]CO_2 Production	
	From [^{14}C]Palmitate	From [^{14}C]Acetate
	dpm/hr per mg protein	*dpm × 10^{-3}/hr per mg protein*
Control (+ lipid)	1,720 ± 130 (n = 2)	707 ± 16
Control (− lipid)	1,110 ± 140	749 ± 2
Cholesterol sulfate (1 μM) (− lipid)	1,650 ± 140	
Cholesterol sulfate (10 μM) (− lipid)	1,680 ± 150	705 ± 11
Cholesterol sulfate (20 μM) (− lipid)	1,620 ± 120	699 ± 20
Cholesterol sulfate (30 μM) (− lipid)	1,550 ± 200	689 ± 9

Preconfluent fibroblast cultures were placed in fresh media plus 10% newborn calf serum (+ lipid) or 10% lipoprotein-depleted serum (− lipid) and vehicle (0.25% methanol; control) or cholesterol sulfate for 24 hr prior to study (see Methods). All assays were performed in triplicate, except "Control (+ lipid)" where n = 2; data are expressed as means ± SEM.

tured fibroblasts (12). In the studies reported here, we have demonstrated a comparable effect of cholesterol sulfate on sterologenesis in cells derived from ectoderm as well, namely the human keratinocyte. The effect of cholesterol sulfate on sterologenesis in both cell types is dependent upon cell density and lipoproteins in the media. That is, in the absence of exogenous lipoproteins, cholesterol synthesis is markedly enhanced in actively expanding cultures of both cell types (26), and it is under these conditions that sterologenesis is most dramatically suppressed by cholesterol sulfate. Nonetheless, cholesterol sulfate is clearly a less potent inhibitor of sterologenesis than either lipoprotein-containing serum or the oxygenated sterol, 25-hydroxycholesterol. We believe that the failure to observe even greater inhibition of sterologenesis with cholesterol sulfate concentrations exceeding 10 μM cannot be ascribed to loss of solubility at higher concentrations, because we continued to observe dose-dependent changes in synthesis of other lipids at these higher concentrations. This observation suggests that cholesterol sulfate may inhibit cholesterol synthesis in a manner different from either oxygenated sterols or serum lipoproteins. In the future, it will be important to determine whether or not cholesterol sulfate affects reductase activity by inhibiting new enzyme synthesis.

Because of the extreme potency of oxygenated sterols as inhibitors of HMG-CoA reductase and in view of their ready generation as auto-oxidation by-products (30), it is difficult to state with absolute certainty that an oxygenated sterol derivative was not the reductase inhibitor in these experiments. Several steps have been taken to address this possibility. First, it was previously shown that our stock cholesterol sulfate possessed only trace impurities and that it was equally inhibitory to sterologenesis as TLC-purified cholesterol sulfate (12). This suggested that if the reductase inhibition were to be attributed to an oxygenated sterol, it was also a sulfated sterol, because only sulfated oxygenated sterols would possess chromatographic mobility similar to cholesterol sulfate. A similar conclusion was suggested by the observation that cholesterol sulfate was a potent inhibitor of sterologenesis in mutant fibroblasts devoid of steroid sulfatase activity (12). In the studies

TABLE 4. Effect of cholesterol sulfate and 25-hydroxycholesterol on incorporation of [^{3}H]oleate into sterol esters in preconfluent and confluent keratinocyte cultures

Cultures	Incorporation into Sterol Esters			
	Acetate		Oleate	
	dpm × 10^{-3}/mg protein			
Preconfluent				
Control	0.49 ± 0.03		10.1 ± 0.7	
Cholesterol sulfate (25 μM)	0.62 ± 0.05	NS	7.1 ± 0.4	$P < 0.01$
25-Hydroxycholesterol (2.5 μM)	2.49 ± 0.16	$P < 0.001$	48.2 ± 1.6	$P < 0.001$
Confluent				
Control	17.5 ± 1.5		149.0 ± 25.4	
Cholesterol sulfate (25 μM)	15.0 ± 5.1	NS	57.0 ± 6.3	$P < 0.02$

Keratinocytes in 10% lipoprotein-depleted fetal calf serum were exposed to cholesterol sulfate, 25-hydroxycholesterol, or vehicle (0.25% methanol; control) for 24 hr. Media and additives were renewed and cultures were incubated with [^{14}C]acetate (7×10^6 dpm/dish) or [^{3}H]oleate (4×10^6 dpm/dish) for an additional 24 hr. Data are means of three dishes ± SEM. Significance was determined by Student's *t*-test; NS, not significant.

reported here, we examined the possibility that inhibitory auto-oxygenated derivatives were generated during cell culture incubations. However, we observed that cholesterol sulfate in the presence of the antioxidant α-tocopherol was *more,* rather than less, inhibitory to sterologenesis. While this latter observation may suggest that α-tocopherol prevented the emergence of noninhibitory oxygenated derivatives of cholesterol sulfate, alternative possibilities, such as an effect on steroid sulfatase activity or cholesterol sulfate transport, must be considered. Finally, the observations that concentrations of cholesterol sulfate exceeding 10 μM did not result in greater inhibition of sterologenesis and that maximal inhibition in response to cholesterol sulfate was significantly less than that in response to either lipoprotein-containing serum or 25-hydroxycholesterol argue somewhat against an oxygenated sterol contaminant as the inhibitor. Taken together, these data indicate that cholesterol sulfate itself, or perhaps a sulfated oxygenated derivative, inhibits sterologenesis. Whether cholesterol sulfate itself inhibits HMG-CoA reductase activity or whether it leads to the intracellular enzymatic generation of an oxygenated sterol which is the active reductase inhibitor, as has been suggested for LDL-cholesterol (31), remains to be determined.

In cells that are capable of LDL receptor-mediated regulation of sterologenesis (e.g., normal and RXLI fibroblasts and preconfluent keratinocytes), cholesterol sulfate inhibited sterologenesis only in lipoprotein-depleted media. In lipoprotein-containing media, sterologenesis in these cells was suppressed and could not be further inhibited by cholesterol sulfate. However, in cells exhibiting high levels of sterologenesis but lacking LDL receptors (e.g., confluent keratinocyte cultures and preconfluent fibroblasts genetically deficient in LDL receptors), cholesterol sulfate inhibited sterologenesis in both lipoprotein-containing as well as lipoprotein-depleted media, although higher concentrations were required in the former media because of partition of cholesterol sulfate to lipoproteins. This lipoprotein-associated cholesterol sulfate would be unavailable to cells not expressing LDL receptors.

In this report we demonstrate for the first time that cholesterol sulfate modulates the metabolism of nonsterol lipids in cultured human fibroblasts and keratinocytes as well. Whereas cholesterol sulfate markedly increased acetate incorporation into fatty acid-containing lipid fractions, these findings cannot be attributed to shifting of acetate pools toward fatty acid synthesis as a consequence of sterologenesis inhibition. This is because: *a*) the effect is not produced with either lipoprotein-containing serum or 25-hydroxycholesterol, both of which more profoundly suppress acetate incorporation into sterol than does cholesterol sulfate; *b*) the dose–response curves for these two effects of cholesterol sulfate on lipid metabolism are dissociated; and *c*) CO_2 production from acetate is unchanged (92% of control). Moreover, because similar effects on fatty acid metabolism are observed in steroid sulfatase-deficient cell lines, this effect on fatty acid metabolism can be attributed to cholesterol sulfate itself, rather than cholesterol following intracellular desulfation. Both keratinocyte and fibroblast cultures are particularly susceptible to cholesterol sulfate when they are in the preconfluent state. More modest increases in acetate incorporation into some fatty acid-containing fractions are observed in confluent fibroblast cultures in response to cholesterol sulfate. In confluent keratinocyte cultures, cholesterol sulfate is inhibitory to acetate incorporation into fatty acid-containing lipids.

It is likely that the effect of cholesterol sulfate on fatty acid metabolism is due to increased synthesis, rather than impaired degradation. This is because: *a*) incorporation of exogenous fatty acid into lipid; *b*) acid lipase activity; and *c*) long-chain fatty acid oxidation are all unaffected by cholesterol sulfate. Since acetyl coenzyme A carboxylase represents the first committed step in fatty acid biosynthesis (reviewed in 32), experiments are underway to determine whether cholesterol sulfate directly affects this enzyme. Nonetheless, it is possible that the effects of cholesterol sulfate on fatty acid synthesis are toxic and may be due to detergent-like effects of this highly amphipathic molecule on cellular membranes and/or membrane-associated enzymes. Another class of amphipathic lipids, synthetic retinoids, has recently been reported to exert similar effects on lipid metabolism in cultured keratinocytes (33). However, the observation that these effects of cholesterol sulfate are modulated by such factors as the state of confluence of the culture and the presence of exogenous lipid argues somewhat against a nonspecific, toxic effect. Whether toxic or physiologic, these effects may be relevant to disease states characterized by accumulation of this sterol metabolite.

Our data also suggest that cholesterol sulfate inhibits sterol esterification in keratinocyte cultures, but not in fibroblasts. Goldstein et al. (29) have suggested that one class of steroids suppresses HMG-CoA reductase while simultaneously inhibiting acyltransferase activity. In their studies, these compounds were inhibitory to esterification in cell-free preparations, implying a direct effect on this enzyme. Effects of cholesterol sulfate on acyltransferase activity have not yet been examined.

Our studies suggest three potential sites of cholesterol sulfate action on cellular lipid metabolism: *a*) inhibition of sterol synthesis; *b*) inhibition of sterol esterification; and *c*) stimulation of fatty acid synthesis. Clearly, these observations on lipid metabolism in vitro raise the possibility that similar effects may be operative in vivo. Thus, the conditions under which cholesterol sulfate has its greatest effects on lipid metabolism in cell culture, i.e., rapidly growing cultures in lipoprotein-depleted media, may not apply to intact tissues normally exposed to serum

lipoproteins. There are several observations, however, which taken together suggest that these effects of cholesterol sulfate may have particular relevance to epidermal lipid metabolism. First, cholesterol sulfate has been shown to be synthesized in mouse epidermis (34) and is present in stratum corneum in significant quantities (7, 8). Secondly, in vivo studies have provided evidence that skin is an extremely active organ of de novo sterologenesis (35, 36). Brannan, Goldstein, and Brown (37) observed very high levels of HGM-CoA reductase activity in hair bulbs, and failure to alter this activity with changes in serum LDL concentrations. Recent evidence suggests that lipid synthesis in epidermis may be attuned to requirements for the provision of the stratum corneum permeability barrier (38) rather than to the availability of serum lipids.

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