Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and cholesterol biosynthesis by oxylanosterols

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Abstract Treatment of rat intestinal epithelial cell cultures with the oxidosqualene cyclase inhibitor, 3β-[2-(diethylamino)-ethoxy]androst-5-en-17-one (U18666A), resulted in an accumulation of squalene 2,3:22,23-dioxide (SDO). When U18666A was withdrawn and the cells were treated with the sterol 14α-demethylase inhibitor, ketoconazole, SDO was metabolized to a product identified as 24(*S*),25-epoxylanosterol. To test the biological effects and cellular metabolism of this compound, we prepared 24(*RS*),25-epoxylanosterol by chemical synthesis. The epimeric mixture of 24,25-epoxylanosterols could be resolved by high performance liquid chromatography on a wide-pore, non-endcapped, reverse phase column. Both epimers were effective suppressors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity of IEC-6 cells. The suppressive action of the natural epimer, 24(*S*),25-epoxylanosterol, but not that of 24(*R*),25-epoxylanosterol could be completely prevented by ketoconazole. IEC-6 cells could efficiently metabolize biosynthetic 24(*S*),25-epoxy[^{3}H]lanosterol mainly to the known reductase-suppressor 24(*S*),25-epoxycholesterol. This metabolism was substantially reduced by ketoconazole. These data support the conclusion that 24(*S*),25-epoxylanosterol per se is not a suppressor of HMG-CoA reductase activity but is a precursor to a regulatory oxysterol(s). It has recently been reported that 25-hydroxycholesterol can occur naturally in cultured cells in amounts sufficient to effect regulation of HMG-CoA reductase (Saucier et al. 1985. *J. Biol. Chem.* **260:** 14571–14579). In order to investigate the biological effects of possible precursors of 25-hydroxycholesterol, we chemically synthesized 25-hydroxylanosterol and 25-hydroxylanostene-3-one. Both oxylanosterol derivatives suppressed cellular sterol synthesis at the level of HMG-CoA reductase. U18666A had the unusual effect of potentiating the inhibitory effect of 25-hydroxylanostene-3-one but did not influence the effect of other oxylanosterols. All the oxylanosterols, with the exception of 25-hydroxylanostene-3-one, enhanced intracellular esterification of cholesterol. The foregoing observations support consideration of oxylanosterols as playing an important role in the biological formation of regulatory oxysterols that modulate sterol biosynthesis at the level of HMG-CoA reductase.—**Panini, S. R., R. C. Sexton, A. K. Gupta, E. J. Parish, S. Chitrakorn, and H. Rudney.** Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and cholesterol biosynthesis by oxylanosterols. *J. Lipid Res.* 1986. **27:** 1190–1204.

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The rate of sterol biosynthesis in a variety of mammalian cells is regulated at the level of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by exogenous low density lipoprotein cholesterol (1). While this regulatory property is not exhibited by purified nonlipoprotein cholesterol (2–5), several oxygenated derivatives of cholesterol and lanosterol are potent suppressors of sterol biosynthesis in cultured cells (2, 3, 6–8), leading to the hypothesis that oxysterols may be the natural regulators of cholesterol synthesis in the intact cell (3). It has been suggested that such regulatory oxysterols may arise endogenously from internalized cholesterol by either nonenzymic or controlled enzymic oxidation, or from biosynthetic lanosterol as obligatory precursors of cholesterol during cytochrome P_{450}-dependent 14α-demethylation (3, 6, 7).

An alternate pathway for the endogenous formation of oxysterols as by-products of sterol biosynthesis has been described (9–15). Such compounds are derived from squalene 2,3-epoxide by the introduction of a second oxygen function prior to cyclization. Thus, the important intermediate in this pathway is squalene 2,3:22,23-dioxide

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; SDO, squalene 2,3:22,23-dioxide; 24(*S*),25-epoxylanosterol, *S*-EL, 24(*S*),25-epoxy-5α-lanost-8-en-3β-ol; 24(*R*),25-epoxylanosterol, *R*-EL, 24(*R*),25-epoxy-5α-lanost-8-en-3β-ol; 25-hydroxylanosterol,HL, 5α-lanost-8-ene-3β,25-diol; 25-hydroxylanostene-3-one, 5α-lanost-8-ene-25-ol-3-one; LPDS, lipoprotein-deficient fetal bovine serum (d > 1.21); U18666A, 3β-[2-(diethylamino)ethoxy]-androst-5-en-17-one; ACAT, acyl-CoA:cholesterol acyltransferase; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.

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(SDO). While under normal conditions SDO is a minor by-product of the sterol synthetic pathway, inhibitors of oxidosqualene cyclase such as 4,4,10β-trimethyl-*trans*-decal-3β-ol or 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A) cause an accumulation of this compound in cultured cells (10, 14). In the absence of inhibitors, SDO is metabolized to polar products (10, 14), with concomitant suppression of HMG-CoA reductase activity (15). The product of SDO metabolism in rat liver, under aerobic conditions, has been identified as 24(*S*),25-epoxycholesterol (12, 13). Recent work by Nelson, Steckbeck, and Spencer (13), Spencer et al. (16), and Saucier et al. (17) has suggested that 24(*S*),25-epoxycholesterol may not only be a natural product of mammalian sterol synthesis, but may also play a role in the regulation of HMG-CoA reductase activity. This compound was shown to be an effective suppressor of reductase activity in mouse L cells and in Chinese hamster lung fibroblasts and, in addition, was present in these cell lines in detectable amounts (16, 17).

The product of anaerobic metabolism of SDO in yeast as well as in rat liver is 24(*S*),25-epoxylanosterol (9, 12, 13). In brief reports, we (18) and others (19) have observed that chemically synthesized 24,25-epoxylanosterol, which is an epimeric mixture of the natural 24(*S*)- and the unnatural 24(*R*)-forms, is an effective suppressor of HMG-CoA reductase activity in cultured cells. Rachal et al. (19) reported that 24,25-epoxylanosterol also inhibited reductase activity in a mutant cell line deficient in 14α-demethylase activity. In addition, suppression of reductase activity in this cell line by lipoproteins in normal serum was associated with rapid cellular synthesis of a compound with chromatographic properties of 24,25-epoxylanosterol. These results were interpreted to mean that endogenous 24,25-epoxylanosterol directly mediates regulation of HMG-CoA reductase activity. On the other hand, studies from our laboratory have demonstrated (20) that treatment of a rat intestinal epithelial cell line with 30 μM ketoconazole, a synthetic inhibitor of sterol 14α-demethylase activity (21, 22), resulted in considerable cellular accumulation of lanosterol and 24(*S*),25-epoxylanosterol, while the activity of HMG-CoA reductase was not inhibited. Interestingly, this level of ketoconazole totally abolished the suppressive effect of low density lipoproteins on HMG-CoA reductase and partially prevented that of exogenous 24,25-epoxylanosterol. These data led us to propose (20) that 24(*S*),25-epoxylanosterol may not be a reductase suppressor per se, but may only be a precursor to an eventual suppressor such as 24(*S*),25-epoxycholesterol. The results with exogenous 24,25-epoxylanosterol may be complicated by the fact that the metabolism of the two epimers is different. In rat liver homogenates, the *S*-form is metabolized to 24(*S*),25-epoxycholesterol, while the *R*-form is converted to 24(*R*)-hydroxycholesterol under aerobic conditions and to 24(*R*)-hydroxylanosterol under anaerobic conditions (23).

In the current work we have attempted to reconcile the foregoing observations by a detailed study of the biological effects and the metabolism of homogeneous 24(*S*),25-epoxylanosterol in IEC-6 cells. We have also studied the effects of two oxylanosterol analogs, 25-hydroxylanosterol and 25-hydroxylanostene-3-one, on sterol biosynthesis and the activity of HMG-CoA reductase in this cell line. It has recently been reported that detectable levels of 25-hydroxycholesterol, a very potent suppressor of reductase activity, could be found to occur naturally in Chinese hamster lung fibroblasts (17). However, its biochemical origin was not established. In view of the fact that 25-hydroxylanosterol could be efficiently metabolized to 25-hydroxycholesterol by rat liver homogenates (12), the possibility exists that the former compound may be a natural precursor of the latter in other biological systems as well. Similarly, the natural occurrence of significant levels of C_{30}-sterones in human skin fibroblasts has been observed (24), generating interest in the biological effects of oxygenated derivatives of such sterones. The results of the current study confirm our earlier hypothesis (20) that 24(*S*),25-epoxylanosterol is only an intermediate in the formation of a regulatory oxysterol. In addition, they show that 25-hydroxylanosterol and 25-hydroxylanostene-3-one are potent inhibitors of HMG-CoA reductase activity. Our observations emphasize the important role of oxylanosterol derivatives in physiological regulation of sterol biosynthesis.

EXPERIMENTAL PROCEDURES

General

Melting points were determined with an Electrothermal capillary apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Model 580 spectrometer using KBr pellets. Proton nuclear magnetic resonance (NMR) spectra using $CDCl_3$ as solvent were obtained with a Varian EM-390 spectrometer using tetramethylsilane as an internal standard. Peaks are reported as ppm (δ) downfield from the tetramethylsilane standard. Proton chemical shifts (α) for the C-18 and C-14 angular methyl resonances were measured by the method of Zurcher (25). Mass spectral (MS) analyses were conducted using a DuPont 491 mass spectrometer and the results are presented in terms of relative intensity (% of the base peak) along with probable mode of origin. Optical rotations were recorded using a Rudolph polarimeter with chloroform as solvent. Analytical thin-layer

chromatography (TLC) was performed on plates of silica gel G (Analtech, Newark, DE). Solvent systems (SS) for TLC were as follows: SS-1, chloroform–ether–acetic acid 97:2.5:0.5; SS-2, 50% ether in toluene; SS-3, 50% ethyl acetate in toluene; SS-4, 35% ethyl acetate in chloroform; SS-5, 50% ethyl acetate in hexane; SS-6, 50% ether in hexane; SS-7, chloroform. Components on the plates were visualized by spraying with molybdic acid (26). Gas–liquid chromatographic (GLC) analyses were performed on a Varian 3700 gas chromatograph equipped with dual flame ionization detectors using 3% OV-1 and 3% OV-17 columns at 270°C. Silanized glass columns (6 ft × 2 mm i.d.) were employed using nitrogen as the carrier gas (25 ml/min). Silica gel (60–200 mesh) column chromatography was conducted on columns that were 60 cm in length and 1.5 cm in diameter. Fractions, 20 ml in volume, were collected. Elemental microanalyses were carried out by Galbraith Laboratories Inc. (Knoxville, TN).

Materials

Pyridinium chlorchromate (PCC), *m*-chloroperbenzoic acid (*m*-CPBA) and lithium aluminum hydride ($LiAlH_4$) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Commercial "lanosterol" (ICN Pharmaceuticals, Inc., Cleveland, OH) was recrystallized three times from acetone–water prior to use and was found to be a mixture of lanosterol (40%) and 24,25-dihydrolanosterol (60%) upon GLC (3% OV-17; 250°C) analysis. Sodium [^{3}H]acetate (1.6 Ci/mmol), *RS*-[3-^{14}C]HMG-CoA (57 mCi/mmol), *RS*-[5-^{3}H(N)]mevalonolactone (24 Ci/mmol), [4-^{14}C]cholesteryl oleate (55.5 mCi/mmol), and 25-hydroxy[26,27-^{3}H]cholesterol (87 Ci/mmol) were from New England Nuclear Corp. (Boston, MA). [4-^{14}C]Cholesterol (50 mCi/mmol) and [1-^{14}C]oleic acid (55 mCi/mmol) were from Research Products International (Mount Prospect, IL). [9,10(n)-^{3}H]Oleic acid (4.3 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). Ketoconazole and U18666A were generous gifts from Janssen Pharmaceuticals, Inc., New Brunswick, NJ and Dr. Richard Cenedella, Kirksville College of Osteopathic Medicine, Kirksville, MO, respectively. We are indebted to Dr. Thomas A. Spencer of the Department of Chemistry, Dartmouth College, Hanover, NH for providing us with the sample of 24(*S*),25-epoxycholesterol used as a reference standard in high performance liquid chromatographic (HPLC) analysis of sterols.

Cell cultures

Rat intestinal epithelial cells (IEC-6, CRL-1592), obtained from American Type Culture Collection (Rockville, MD), were grown as monolayers as described before (14, 15).

HMG-CoA reductase activity and sterol synthesis

The activity of HMG-CoA reductase in logarithmically growing cultures was determined as previously described (15). One unit of the enzyme activity equals the formation of 1 pmol mevalonate/min. The results are presented as the average of triplicate determinations at each data point. The variation in data did not exceed 7% of the mean. For the measurement of enzyme activity as well as of lipid synthesis from acetate, 2×10^5 cells were seeded in 60 × 15 mm plastic petri dishes in 2 ml of medium containing 5% (v/v) fetal bovine serum on day 0. On days 3 and 4, the monolayers were refed 2 ml of medium containing 5% (v/v) lipoprotein-deficient serum (LPDS). The LPDS (d > 1.21 g/ml) was prepared from fetal bovine serum by ultracentrifugation using KBr for density adjustment (27). On day 4, groups of dishes were pretreated with indicated concentrations of oxysterols dissolved in 20 μl of ethanol. Control dishes received an equal volume of ethanol. After 5 hr of pretreatment, the monolayers were pulsed with [^{3}H]acetate (10 μCi/dish) for 1 hr. At the end of the incubation, monolayers were rinsed three times with ice-cold saline, drained thoroughly, and the total lipids were extracted by the addition of 5 ml of hexane–isopropanol 60:40 containing carrier amounts of cholesterol, lanosterol, 24,25-epoxylanosterol, ubiquinone, SDO, squalene-2,3-epoxide, squalene, and [4-^{14}C]cholesterol (50,000 dpm) as an internal standard, and incubated at room temperature for 20–30 min. This procedure efficiently extracts the nonsaponifiable lipids from monolayers of cultured cells (28). After removal of the extract, the cell residue remaining on the dishes was digested in 0.1 N NaOH for determination of protein content (15). Nonsaponifiable lipids were isolated from the total lipid extract as previously described (14) and the individual lipid classes were separated by TLC on Whatman K5 silica gel 150 plates using petroleum ether–acetone 90:10 as the developing solvent. The use of wide-pore silica resolved all the lipid components as effectively as the two-dimensional TLC system previously described (14). Typical R_f values were as follows: polar sterols, 0.04–0.22; cholesterol, 0.25; 24,25-epoxylanosterol, 0.31; lanosterol, 0.38; ubiquinone, 0.52; SDO, 0.57; squalene 2,3-epoxide, 0.68; squalene, 0.75. The lipids were visualized by spraying the plates with *p*-anisaldehyde (29). The radioactivity in individual fractions was determined in a Beckman LS-335 liquid scintillation spectrometer (Beckman Instruments, Palo Alto, CA). The results were corrected for the recovery of [^{14}C]cholesterol internal standard. The aqueous phase, after the extraction of nonsaponifiable lipids, was acidified with HCl and the fatty acids were extracted in 20 ml of hexane with 96% efficiency. The concentration of oxylanosterol required to inhibit sterol or total nonsaponifiable lipids synthesis by 50% was

estimated from plots of the ratio of ^{3}H-labeled sterols or ^{3}H-labeled nonsaponifiable lipids to ^{3}H-labeled fatty acids as a function of the concentration of the inhibitor (30).

Esterification of cellular cholesterol

The effect of oxylanosterols on the esterification of cellular cholesterol in IEC-6 cells was determined by measuring the incorporation of exogenous [^{3}H]oleate into cholesteryl esters essentially as described by Goldstein, Dana, and Brown (31). At the end of incubation with [^{3}H]oleate (10 μCi/dish, 100 dpm/pmol), total lipids were extracted with hexane–isopropanol 60:40 containing 25 μg of carrier cholesteryl oleate and 25,000 dpm of [4-^{14}C]cholesteryl oleate and the components were separated by TLC on Merck silica gel 60 plates using hexane–diethyl ether–acetic acid 80:20:1 as the solvent. Lipids were visualized by spraying with *p*-anisaldehyde (29). The spots corresponding to cholesteryl esters were scraped into vials for determination of radioactivity which was appropriately corrected for recovery of the internal standard.

Chemical synthesis of oxylanosterols

Chemical synthesis of oxylanosterol derivatives was accomplished by a direct route using recrystallized commercial "lanosterol" (a mixture of lanosterol and 24,25-dihydrolanosterol) as the starting material (**Fig. 1**).

24,25-Epoxy-5α-lanost-8-en-3β-ol (24,25-epoxylanosterol)

To a solution of recrystallized commercial lanosterol (25 g) in CH_2Cl_2 (2500 ml), $NaHCO_3$ (2 g) and *m*-CPBA (4 g) were successively added. After stirring for 24 hr at 25°C, the mixture was washed with 1 N NaOH and water and dried over anhydrous $MgSO_4$. Analysis by TLC (SS-1) showed two major components with R_f values of 0.55 and 0.51. The less polar component had the same mobility as lanosterol and 24,25-dihydrolanosterol. The more polar component had the mobility of 24,25-epoxylanosterol (vide infra). The residue obtained upon evaporation of solvent under reduced pressure was recrystallized from

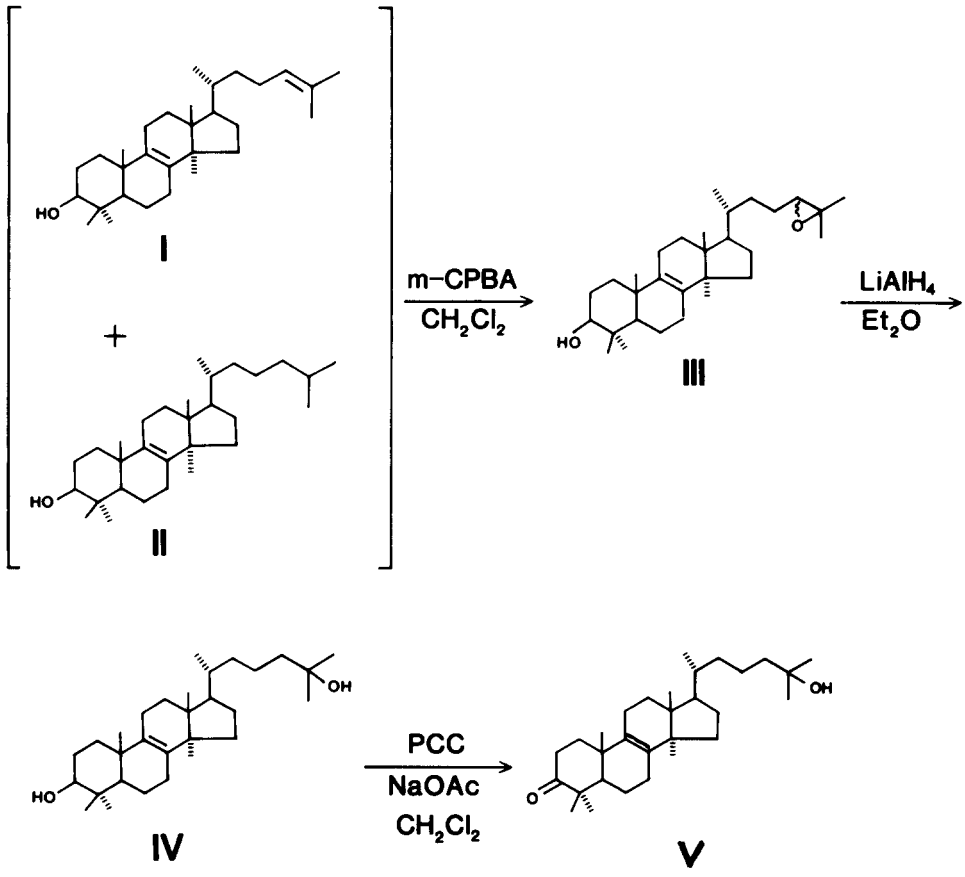

Fig. 1. Chemical synthesis of oxylanosterols. Monoepoxidation of recrystallized mixture of lanosterol (I) and 24,25-dihydrolanosterol (II) with *m*-chloroperbenzoic acid yielded an epimeric mixture of 24,25-epoxylanosterols (III). Purified 24,25-epoxylanosterol could be converted to 25-hydroxylanosterol (IV) by treatment with $LiAlH_4$. Oxidation of 25-hydroxylanosterol with buffered pyridinium chlorchromate produced 25-hydroxylanostene-3-one (V). Details of these procedures are given in the Experimental Procedures section.

CH_2Cl_2–CH_3OH by partial removal of CH_2Cl_2 under a stream of N_2, cooling to −15°C and filtering to remove lanosterol and 24,25-dihydrolanosterol. Repetition of this procedure (seven times) produced a concentrated solution containing approximately 95% of 24,25-epoxylanosterol by TLC (SS-1) analysis. The enriched mixture was subjected to silica gel column chromatography using a solvent gradient of diethyl ether (0–25%) in toluene. The contents of the column fractions containing the major product were pooled and, after evaporation of the solvent, recrystallized from acetone–water to yield 2.75 g of 24,25-epoxylanosterol; m p 119–123°C; IR, 3460, 1650, and 1030 cm^{-1}; NMR, 0.68 (s, 3H, C–18–CH_3), 0.88 (s, 3H, C–19–CH_3), 1.28 and 1.30 (2s, 6H, C–26 and 27–CH_3), 2.70 [m, 1H, C–24–H; lit., 2.70 (32)], 4.56 (t, 1H, C–3–H, J < 7 Hz); MS, 442 (19%; M), 427 (29%), M–CH_3), 424 (15%, M–H_2O), 409 (10%, M–CH_3H_2O), 313 (10%, M–side chain), 295 (8%, M–H_2O–side chain). TLC analysis in seven solvent systems (SS-1 through SS-7) showed a single unresolved component. However, this racemic mixture of 24(*S*),25-epoxylanosterol and 24(*R*),25-epoxylanosterol could be resolved by HPLC using a non-endcapped, reverse phase (C-18) column as described later in this section.

5α-Lanost-8-ene-3β,25-diol (25-hydroxylanosterol)

To a solution of 24,25-epoxylanosterol in anhydrous ether (1.5 g or 3.37 mmol in 150 ml), $LiAlH_4$ (3.0 g; 7.9 mmol) was added and the mixture was stirred at 25°C for 8 hr. After cooling to 0°C, ice was cautiously added to decompose the excess hydride. The resulting mixture was poured into saturated aqueous NH_4Cl and thoroughly extracted with 10% CH_2Cl_2 in ether. The combined extracts were dried over $MgSO_4$ and evaporated to dryness under reduced pressure to yield a white solid (1.42 g). The residue was subjected to silica column chromatography using a gradient of ethyl acetate (0–40%) in toluene. Evaporation of solvent and recrystallization from acetone–water yielded 1.33 g (88% yield) of 25-hydroxylanosterol; m p 183.5–185°C [lit., 184–186°C (33)]; IR, 3350, 1640, 1024 and 935 cm^{-1}; NMR, 0.69 [s, 3H, C–18–CH_3, lit., 0.69 (33)], 0.88 [s, 3H, C–19–CH_3, lit., 0.88 (33)], 1.20 [2s, 6H, C–26 and 27–CH_3, lit., 1.20 (33)], 3.22 (m, 1H, C–3–H); MS, 444 (8%, M), 429 (15% M–CH_3), 426 (12%, M–H_2O), 411 (62%, M–CH_3–H_2O), 408 (18%, M–$2H_2O$), 393 (100% M–CH_3–$2H_2O$), 297 (16%, M–H_2O–sidechain). $[\alpha]_D$, +58° (C, 1.19) [lit., +59° (33)]. TLC analysis (SS-1 through SS-7) showed a single component.

5α-Lanost-8-ene-25-ol-3-one (25-hydroxylanostene-3-one)

To a solution of 25-hydroxylanosterol in CH_2Cl_2 (500 mg or 1.12 mmol in 50 ml) molecular sieves (250 mg, type 3A) and sodium acetate (250 mg) were added. After stirring for 15 min, 3 g of pyridinium chlorchromate (34) was added and the mixture was stirred for an additional 1 hr. The reaction mixture was then evaporated under reduced pressure to approximately 20% of its original volume. A saturated NaCl solution was then added and the mixture was thoroughly extracted with ether. The combined extracts were filtered through anhydrous $MgSO_4$ and evaporated to dryness. The residue was subjected to silica column chromatography using a gradient of ether (0–25%) in toluene. Evaporation of solvent and recrystallization from acetone–water provided 428 mg (86% yield) of 25-hydroxylanostene-3-one; m p 138–140°C; IR, 3500, 1705, 1101, and 931 cm^{-1}; NMR, 0.72 (s, 3H, C–18–CH_3), 0.89 (s, 3H, C–19–CH_3), 1.13 (2s, 6H, C–26 and 27–CH_3); MS, 442 (8%, M), 427 (5%, M–CH_3), 424 (29%, M–H_2O), 409 (100%, M–CH_3–H_2O), 313 (5%, M–side chain), 271 (20%), 257 (57%), and 245 (37%). TLC analysis (SS-1 through SS-7) indicated a single component. Elemental analysis yielded C, 81.37%; H, 11.36% ($C_{30}H_{50}O_2$ requires C, 81.39%; H, 11.38%).

Preparation of 24(*S*),25-epoxyl[^{3}H]lanosterol

When IEC-6 cells were incubated with [^{3}H]acetate, less than 0.1% of the radioactivity in the nonsaponifiable lipid extract comigrated with 24,25-epoxylanosterol on TLC (data not shown). In order to maximize the accumulation of 24(*S*),25-epoxylanosterol, we devised a strategy based on our previous observations that treatment of IEC-6 cells with U18666A caused an accumulation of SDO (14) and that a similar treatment with ketoconazole inhibited the metabolism of C_{30}-sterols (20). Thus, IEC-6 cells were incubated with U18666A (2μg/ml) and [^{3}H]acetate (10 μCi/dish) for 24 hr, followed by a refeeding with medium containing 30 μM ketoconazole for 6–24 hr. We reasoned that the SDO that accumulates during the initial treatment of cells with U18666A would be cyclized to 24(*S*),25-epoxylanosterol upon withdrawal of the drug. Subsequent treatment with ketoconazole should then cause an accumulation of 24(*S*),25-epoxylanosterol.

As shown in **Table 1,** after treatment of IEC-6 cells with U18666A, SDO was the major nonsaponifiable lipid product of [^{3}H]acetate metabolism (63–70% of total radioactivity). The radiolabel recovered in 24,25-epoxylanosterol was <2% of total. When these cells were refed fresh medium containing U18666A, the distribution of radiolabel was not altered. However, when the medium contained ketoconazole in the place of U18666A, the amount of ^{3}H associated with the 24,25-epoxylanosterol fraction increased dramatically to comprise 16–25% of the total incorporation. In the absence of both U18666A and ketoconazole, SDO was metabolized instead to polar sterols. The 24,25-epoxylanosterol zones from several TLC fractionations were pooled and eluted with ethyl-

TABLE 1. Biosynthesis of 24(S),25-epoxylanosterol in IEC-6 cells

	^{3}H-Labeled Fraction as % of Total			
		6 Hr after Refeeding		
	24 Hr U18666A	U18666A	KC	LPDS
Experiment 1				
Polar sterols	18.7	19.3	18.5	31.6
Cholesterol	2.8	2.5	2.6	3.6
S-EL	1.6	1.8	16.9	1.9
SDO	69.8	70.4	55.7	56.0
Experiment 2				
Polar sterols	18.0	20.5	20.5	36.3
Cholesterol	4.4	4.4	5.0	5.6
S-EL	1.7	1.9	24.6	2.5
SDO	63.9	64.9	43.0	47.5

IEC-6 cells on day 4 were refed fresh LPDS medium containing U18666A (2 μg/ml). After 1 hr, [^{3}H]acetate (10 μCi/dish) was added. Triplicate dishes were harvested after 24 hr for the determination of radioactivity incorporated into nonsaponifiable lipids as described in the Experimental Procedures section. Remaining dishes were divided into three groups and refed fresh LPDS medium containing either U18666A (2 μg/ml) or ketoconazole (KC, 30 μM) or no addition, to determine the redistribution of incorporated radioactivity during the following 6 hr. Total (100%) incorporation of ^{3}H in 24 hr averaged 2.8×10^6 dpm/mg of protein. The results of redistribution of radiolabel among nonsaponifiable lipids are the mean of triplicate determinations. The variation in data did not exceed 5% of the mean. *S*-EL, 24(*S*),25-epoxylanosterol; SDO, squalene 2,3:22,23-dioxide.

acetate. 24(*S*),25-Epoxy[^{3}H]lanosterol was further purified and quantitated by HPLC as described below. In a typical preparation, the yield of 24(*S*),25-epoxy[^{3}H]-lanosterol was 0.5 pg/cell with a specific radioactivity of 82 μCi/μmol.

Separation of epimers of 24,25-epoxylanosterol by HPLC

The 24,25-epoxylanosterol prepared by chemical synthesis remained a mixture of the natural (*S*)-form and the unnatural (*R*)-form despite extensive silica gel column chromatography and TLC. Since the metabolism of the two epimers in rat liver homogenates is reported to be different (23), it is possible that the use of epimeric mixture in IEC-6 cells could also result in the formation of two different end products (and corresponding intermediates) with different biological effects. Published procedures for the separation of acetate derivatives of 24,25-epoxylanosterol (33, 35) did not yield satisfactory results in our hands. Subsequently, we discovered that the two epimers could be resolved by nonaqueous reverse phase HPLC. A Gilson HPLC system (Gilson Electronics, Middleton, WI) using an Apple *IIe* computer (Apple Computer Co., Cupertino, CA) as the system controller and a data analysis software package capable of integrating data from a Flo-One model HP flow-through radioactivity detector (Radiomatic Instruments, Inc., Tampa, FL) and a V^4 absorbance detector (Isco, Inc., Lincoln, NE) operating at 210 nm was used. The separation of stereoisomers of 24,25-epoxylanosterol was carried out on a Vydac (Separations Group, Hesperia, CA) 201TP54 reverse phase C18 analytical column (0.46 × 25 cm) eluted with either acetonitrile–isopropanol 80:20 or 100% methanol (1 ml/min). The separated epimers were collected using a Gilson model 202B fraction collector operating in the peak detection mode. Fractions pooled from several runs were concentrated and purified by reinjection until homogeneous. The epoxylanosterols were quantified by integration of areas under A_{210} peaks. The two epimers had identical A_{210} responses.

The epimeric mixture of 24,25-epoxylanosterols resolved into two peaks with retention times (RT) of 11.6 and 12.7 min upon HPLC on a wide-pore non-endcapped C18 column (**Fig. 2**). The material from the slower peak (RT = 12.7 min) had a melting point (131–134°C) that was identical to the literature value for 24(*R*),25-epoxylanosterol (33). Consequently, the material from the faster peak (RT = 11.6 min., m p 120–123°C) was designated as 24(*S*),25-epoxylanosterol. This designation was confirmed by the fact that biosynthetically prepared 24,25-epoxy[^{3}H]lanosterol, which occurs only in the *S*-form (12, 13, 23), coeluted with the faster peak (Fig. 2A, B). When the separated epoxides, pooled from several runs, were treated with $LiAlH_4$, each was quantitatively converted to a compound that coeluted (RT = 8.0 min) with 25-hydroxylanosterol (Fig. 2D). Similarly, biosynthetic 24(*S*),25-epoxy[^{3}H]lanosterol could be reduced to 25-hydroxy[^{3}H]lanosterol (Fig. 2D).

HPLC analysis of sterols

For analysis of products of cellular metabolism of 24(*S*),25-epoxy[^{3}H]lanosterol, a Zorbax (DuPont Industries, Wilmington, DE) ODS column (0.46 × 25 cm) was employed. At the end of incubation of IEC-6 cells with 24(*S*),25-epoxy[^{3}H]lanosterol (0.4 μM, 160,000 dpm/dish), the monolayers were rinsed three times with ice-cold saline, and total lipids were extracted with hexane–isopropanol 60:40. Nonsaponifiable lipids isolated from the total lipid extract as described before (14), were dissolved in hexane–chloroform 19:1 and passed through a Prep-Sep silica cartridge (Fisher Scientific Co., Cincinnati, OH) essentially as described by Saucier et al. (17). The sterol fraction was eluted in acetone and the solvent was evaporated under N_2. The residue was dissolved in methanol and, after filtration through a 0.45μm Acro LC3S filter (Gelman Sciences, Inc., Ann Arbor, MI), was concentrated for injection into the HPLC column. Sterols were identified by their elution in 100% methanol (1 ml/min) at the retention times of authentic standards: 25-hydroxycholesterol, 6.0 min; 25-hydroxylanosterol, 7.0 min; 24(*S*),25-epoxycholesterol, 8.0 min; 24(*S*),25-epox-

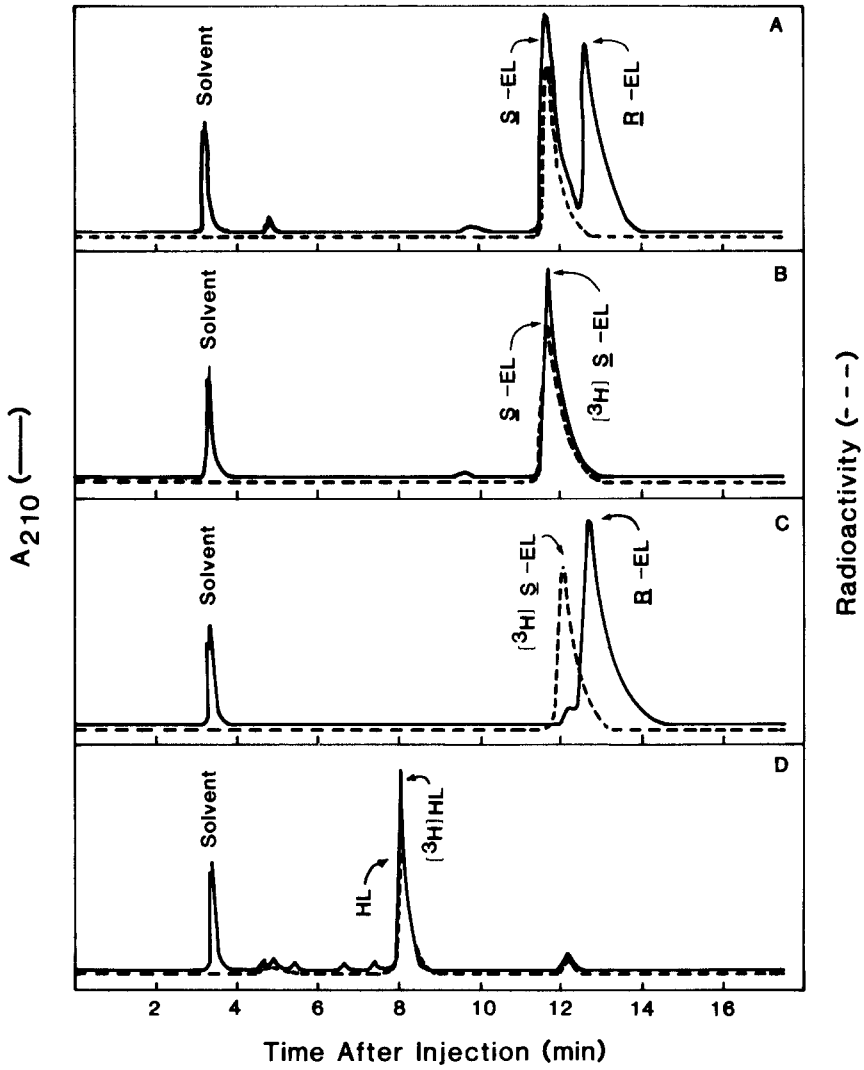

Fig. 2. Separation of 24,25-epoxylanosterol epimers by HPLC. The epimeric mixture of 24,25-epoxylanosterols was separated by HPLC on a Vydac TP201 reverse phase column (0.46 × 25 cm) developed with 100% methanol (1 ml/min). Usually, 20–100 μg of the mixture was injected and the separated peaks were collected (panel A). Pooled peaks from several runs were concentrated and purified by reinjection at least twice. In the end, peak 1 (RT = 11.6 min) was >99% pure (panel B), and peak 2 (RT = 12.7 min) was 95% pure (panel C) as judged by integration of areas under A_{210} peaks. 24(*S*),25-Epoxy[^{3}H]lanosterol ([^{3}H]*S*-EL), isolated by TLC from drug-treated cells as described in Table 1, had a retention time identical to that of peak 1 when injected with the epimeric mixture (panel A) or with either peak (panels B and C). When peak 1 from panel B was treated with $LiAlH_4$ in anhydrous ether and the extracted material was rechromatographed, a single major peak with retention time (RT = 8.0 min) identical to that of 25-hydroxylanosterol (HL, [^{3}]HL), was observed (panel D). This peak accounted for >85% of total A_{210} area as well as of injected radioactivity. Peak 2 material from panel C yielded a similar result after $LiAlH_4$ reduction (data not shown).

ylanosterol, 9.6 min; desmosterol, 17.5 min; lanosterol, 21.5 min; and cholesterol, 22.6 min.

RESULTS

Effect of oxylanosterols on HMG-CoA reductase activity and sterol synthesis in IEC-6 cells

IEC-6 cells in LPDS medium were treated with varying concentrations of oxylanosterols for 6 hr. The effects of 24(*S*),25-epoxylanosterol, 25-hydroxylanosterol, and 25-hydroxylanostene-3-one on HMG-CoA reductase activity and the incorporation of [^{3}H]acetate into C_{27}-sterols and fatty acids are shown in **Fig. 3.** All three compounds were potent suppressors of HMG-CoA reductase activity and inhibited the synthesis of C_{27}-sterols over the same concentration range. None of the compounds had any significant effect on fatty acid synthesis, indicating that they are not general inhibitors of metabolism. These compounds also did not affect general protein synthesis as measured by the incorporation of [^{3}H]leucine into total trichloroacetic acid-precipitable material (data not shown).

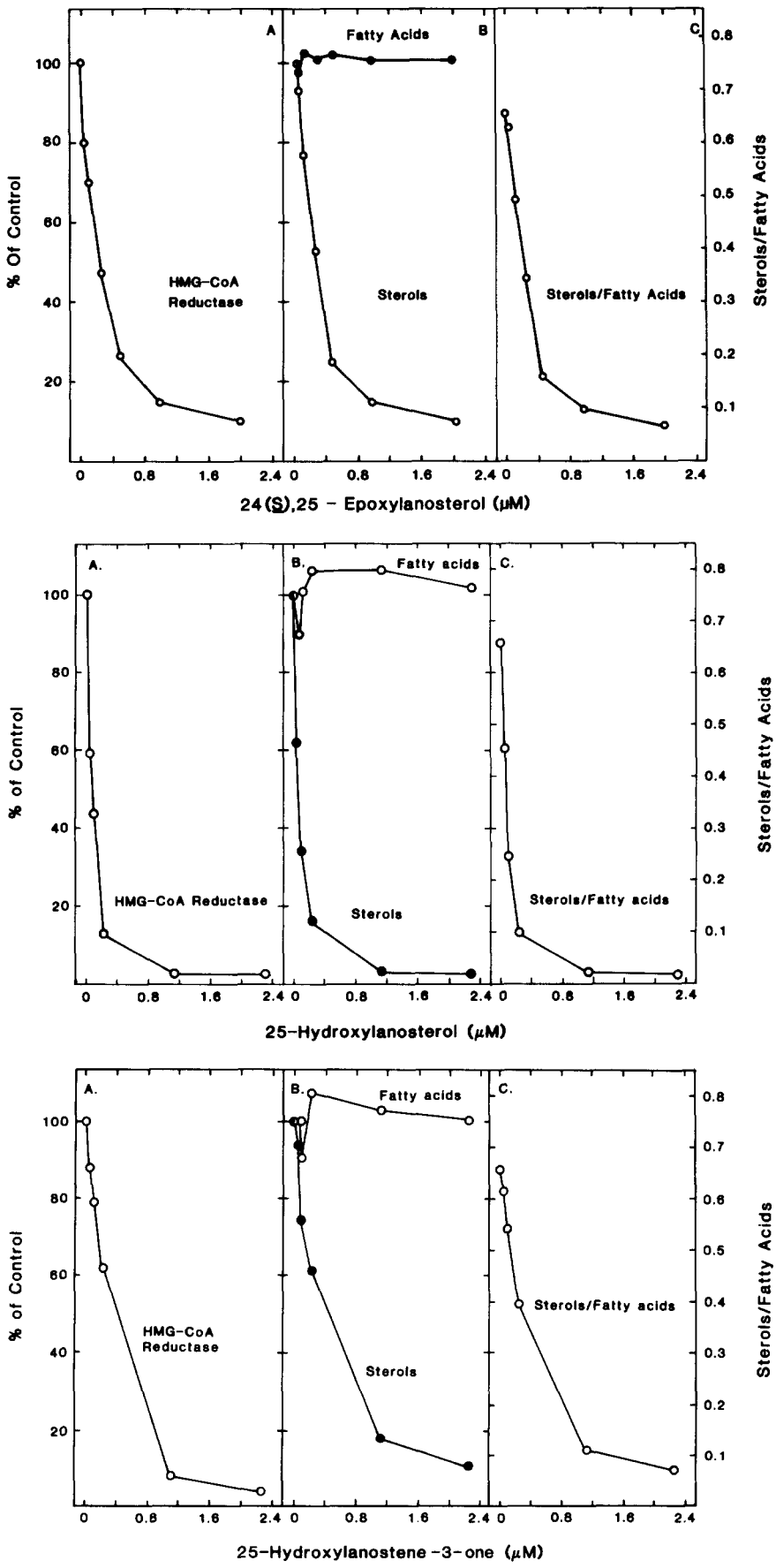

Fig. 3. Effect of 24(*S*),25-epoxylanosterol (top), 25-hydroxylanosterol (middle), and 25-hydroxylanostene-3-one (bottom) on the activity of HMG-CoA reductase (A), incorporation of radiolabel from [^{3}H]acetate into C_{27}-sterols and fatty acids (B), and the ratio of ^{3}H-labeled sterols to ^{3}H-labeled fatty acids (C) in IEC-6 cells. Cells were grown for 4 days as described in the Experimental Procedures section. Groups of dishes in triplicate were treated with varying concentrations of oxylanosterols for 6 hr. Incorporation of [^{3}H]acetate (10 μCi/dish) into C_{27}-sterols and fatty acids was determined during the sixth hour of treatment. Mean control (100%) values for HMG-CoA reductase activity (U/mg of protein), C_{27}-sterol synthesis (dpm × 10^{-5}/hr per mg of protein), and fatty acid synthesis (dpm × 10^{-5}/hr per mg of protein) were 207, 1.40, and 2.12, respectively.

The inhibitory potencies of oxylanosterols are presented in **Table 2.** The concentrations of oxylanosterols required for 50% supression (I_{50} values) of HMG-CoA reductase activity (0.10–0.30 μM), of C_{27}-sterol synthesis (0.09–0.29 μM) and of total nonsaponifiable lipid synthesis (0.11–0.29 μM) were very similar. It is clear from the data that the extent of supression of HMG-CoA reductase corresponds well with the degree of observed inhibition of the synthesis of both C_{27}-sterols and total nonsaponifiable lipids. These data suggest that the oxylanosterols do not exhibit a secondary site of inhibition in the sterol biosynthetic pathway beyond the step catalyzed by HMG-CoA reductase.

Of the oxylanosterol derivatives, 25-hydroxylanosterol was two to three times more potent than 24(*S*),25-epoxylanosterol and 25-hydroxylanostene-3-one in its effect on all three parameters (Table 2). The latter compound differs from 25-hydroxylanosterol only in the substitution on carbon-3. This decrease in inhibitory potency upon substitution of the 3β-hydroxy group with a 3-keto group is consistent with previous observations (8, 30) regarding the structural requirements of inhibitory oxysterols. Under similar incubation conditions, the I_{50} value for 25-hydroxycholesterol with respect to HMG-CoA reductase activity was 0.06 μM, while that for 24(*R*),25-epoxylanosterol was 0.19 μM. None of the oxylanosterols had any effect on the enzyme activity when added to HMG-CoA reductase assay mixtures of cell extracts at levels up to 150 μM (data not shown).

The inhibitory potencies of oxidized lanostene derivatives compare very well with those of oxidized cholestene derivatives (8), suggesting that both sets of compounds may act by a similar mechanism. It is well established that oxidized derivatives of cholesterol inhibit HMG-CoA reductase activity by a suppression of enzyme synthesis and an enhancement of enzyme degradation, thus causing a very rapid loss of enzyme activity (6). It was, therefore, of interest to determine whether the suppression of reductase activity by oxylanosterol derivatives occurred with similar rapidity.

TABLE 2. Inhibitory potencies of oxylanosterol

Oxylanosterol	Concentration (μM) Required for 50% Inhibition of: Synthesis of Nonsaponifiable Lipids	Synthesis of C_{27}-Sterols	HMG-CoA Reductase Activity
24(*S*)-25,-Epoxylanosterol	0.29	0.25	0.24
25-Hydroxylanosterol	0.11	0.09	0.10
25-Hydroxylanostene-3-one	0.26	0.29	0.30

Inhibitory potencies were calculated from the data in Fig. 3 as described in Experimental Procedures.

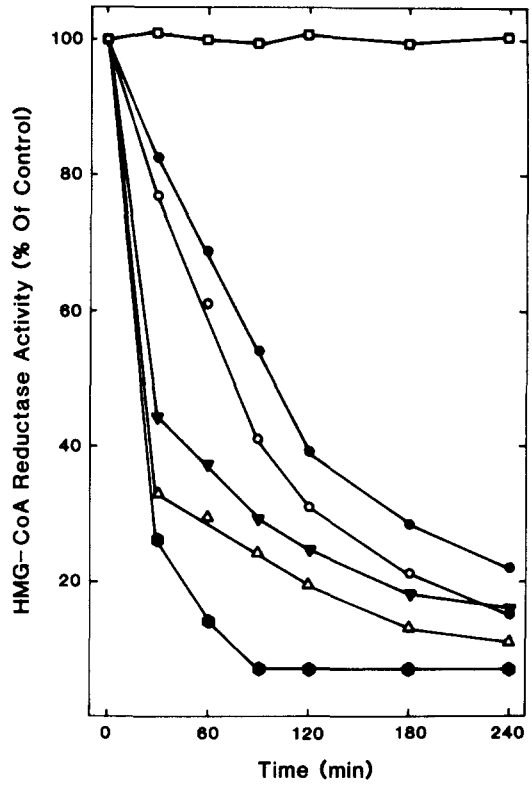

Fig. 4. Time-course of decline in HMG-CoA reductase activity upon incubation of IEC-6 cells with oxylanosterols. Cells were treated with 20 μl of ethanol containing no addition (□, control); 24(*R*),25-epoxylanosterol (●); 24(*S*),25-epoxylanosterol (○); 25-hydroxylanostene-3-one (▼); 25-hydroxylanosterol (△); or 25-hydroxycholesterol (●) for the indicated periods of time and triplicate dishes were harvested for the assay of HMG-CoA reductase activity. The final concentration of each oxysterol in the incubation medium was 1 μM. Control (zero time) value for enzyme activity was 210 U/mg of protein.

Time-course of decline in HMG-CoA reductase activity of IEC-6 cells upon the addition of oxylanosterols

In the absence of inhibitors, the activity of HMG-CoA reductase in IEC-6 cells remained essentially constant over a period of 4 hr (**Fig. 4**). Addition of 25-hydroxylanostene derivatives caused a rapid decline in the enzyme activity. The rate of decrease in enzyme activity was similar to that caused by the well-studied inhibitor, 25-hydroxycholesterol. The time required for a 50% decline in HMG-CoA reductase activity ($t_{1/2}$ value) averaged under 30 min for this group. On the other hand, the $t_{1/2}$ values for 24(*S*),25-epoxylanosterol (75 min) and 24(*R*),25-epoxylanosterol (95 min) were considerably longer. This finding suggested that the uptake of epoxysterols by the cells may be slower when compared to hydroxysterols due to possible differences in solubility. However, a comparison of the uptakes of 24(*S*),25-epoxy[^{3}H]lanosterol and 25-hydroxy[^{3}H]-cholesterol by IEC-6 cells over a similar time-course did not reveal a major difference (data not shown). Alterna-

tively, the action of epoxy derivatives may require prior metabolism to inhibitory products. This possibility was tested further through the use of ketoconazole.

Effect of ketoconazole on the inhibition of HMG-CoA reductase activity by 24,25-epoxylanosterols

It has been reported that, under aerobic conditions, rat liver homogenates can efficiently metabolize 24(*S*),25-epoxylanosterol to 24(*S*),25-epoxycholesterol (12, 13, 23), whereas, the unnatural isomer, 24(*R*),25-epoxylanosterol, is metabolized instead to 24(*R*)-hydroxycholesterol (23). As noted in Table 1, ketoconazole caused an accumulation of 24(*S*),25-epoxylanosterol in IEC-6 cells by preventing the 14α-demethylation of C_{30}-sterols. This drug completely protected the HMG-CoA reductase activity of IEC-6 cells against concentrations of 24(*S*),25-epoxylanosterol up to 0.25 μM (**Fig. 5A**). At this level of 24(*S*),25-epoxylanosterol, the enzyme activity was inhibited by approximately 50% in the absence of ketoconazole. This observation supports the idea (20) that metabolism of 24(*S*),25-epoxylanosterol is a prerequisite to its suppressive action on HMG-CoA reductase. At oxylanosterol concentrations that caused >50% suppression of reductase activity, ketoconazole was unable to fully protect the enzyme activity.

In the case of 24(*R*),25-epoxylanosterol, the protective effect of ketoconazole was only partial at all concentrations of the inhibitor (Fig. 5B). This inferior protection by ketoconazole of the suppressive action of 24(*R*),25-epoxylanosterol suggests that either the *R*-form itself is a suppressor of reductase activity or that it may be metabolized through a ketoconazole-insensitive step such as a reduction to 24(*R*)-hydroxylanosterol (23) which is likely to be a suppressor.

Effect of ketoconazole on the uptake and metabolism of 24(*S*),25-epoxylanosterol in IEC-6 cells

The possibility that ketoconazole may have prevented reductase suppression by 24(*S*),25-epoxylanosterol through an inhibition of its cellular uptake was examined by incubating IEC-6 cells with 24(*S*),25-epoxy[^{3}H]-lanosterol in the presence and absence of the drug. The results presented in **Fig. 6A** demonstrate that ketoconazole in fact enhanced cell-associated radioactivity by approximately 50% at all time points tested. Cellular sterols were also extracted and analyzed by HPLC. As seen in Fig. 6B, in the absence of ketoconazole the radiolabel recovered in 24(*S*),25-epoxylanosterol fraction decreased rapidly. Concurrently, there was a time-dependent increase in the radioactivity associated with a fraction with the retention time of 24(*S*),25-epoxycholesterol. The identity of this material was confirmed by its reduction to 25-hydroxy[^{3}H]cholesterol upon $LiAlH_4$ treatment. In the presence of ketoconazole, the metabolism of 24(*S*),25-epoxylanosterol was substantially prevented. Nevertheless, small amounts of radiolabel (2–5%) could still be detected in the 24(*S*),25-epoxycholesterol peak. When 24(*S*),25-epoxy[^{3}H]lanosterol was similarly incubated for 6 hr in the absence of any cells, >99% of the radiolabel was recovered as unaltered starting material (data not shown).

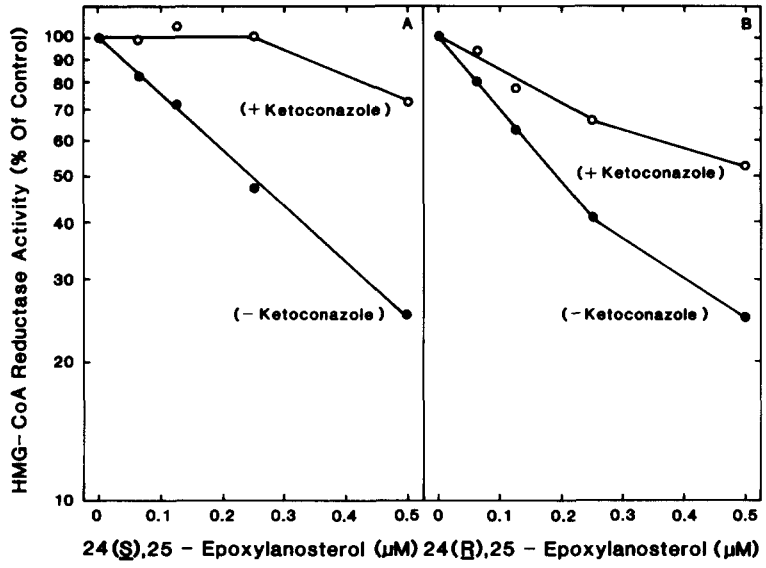

Fig. 5. Effect of ketoconazole on the suppression of HMG-CoA reductase activity of IEC-6 cells by 24(*S*),25-epoxylanosterol (A) and 24(*R*),25-epoxylanosterol (B). Cells were incubated for 1 hr with either no addition (●) or with 30 μM ketoconazole (○). Oxylanosterols were added at the indicated concentrations and triplicate dishes were harvested for the assay of HMG-CoA reductase activity after 6 hr. Mean control (100%) values for enzyme activity in the absence and presence of ketoconazole were 230 and 247 U/mg of protein, respectively.

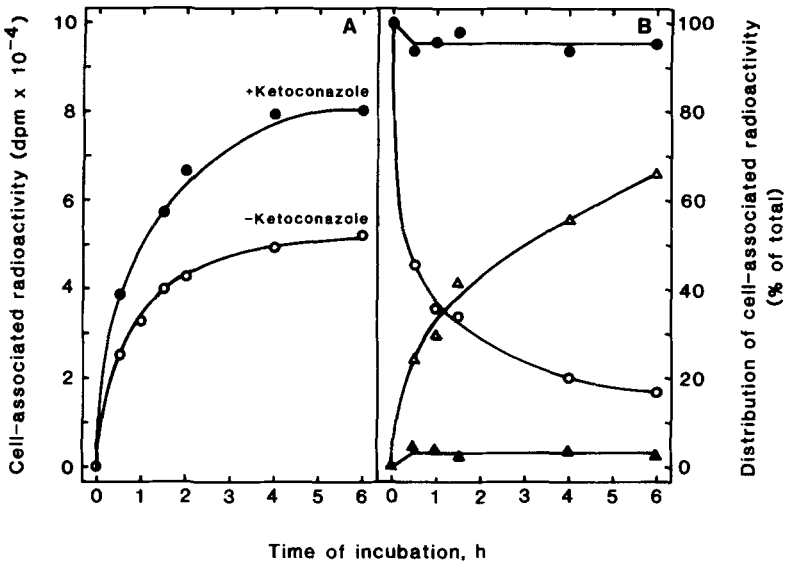

Fig. 6. Effect of ketoconazole on the uptake and metabolism of 24(*S*),25-epoxy[^{3}H]lanosterol in IEC-6 cells. For the study of cellular uptake of oxylanosterol (A), IEC-cells were pretreated for 1 hr with either no addition or with 30 μM ketoconazole. 24(*S*),25-Epoxy[^{3}H]lanosterol (0.4 μM, 160,000 dpm) was then added. At indicated time intervals, triplicate dishes from each group were rinsed three times with saline and cellular sterols were extracted with hexane–isopropanol (60:40). A portion of the extract was used for the determination of cell-associated radioactivity (dpm/dish) in the presence (●) or absence (○) of ketoconazole. The remaining extract was saponified and the nonsaponifiable lipid extract was subjected to reverse phase HPLC on a Zorbax ODS analytical column (0.46 × 25 mm) as described in the Experimental Procedures section. The percent of total radioactivity applied to the column recovered in 24(*S*),25-epoxylanosterol (○, ●) and 24(*S*),25-epoxycholesterol (△, ▲) fractions is presented as a function of the time of incubation (B). Open and filled symbols represent the absence and presence of ketoconazole, respectively.

In addition to 24(*S*),25-epoxycholesterol, more polar products of 24(*S*),25-epoxylanosterol metabolism were also observed, albeit in minor amounts (**Fig. 7**). These fractions were not further characterized. They could be intermediates between 24(*S*),25-epoxylanosterol and 24(*S*),25-epoxycholesterol since their amounts tended to decrease with time. However, such fractions could be observed even in the presence of ketoconazole. It was interesting to note that these fractions had retention times similar to 25-hydroxycholesterol and 25-hydroxylanosterol. The occurrence of biosynthetic 25-hydroxycholesterol in Chinese hamster lung fibroblasts has been reported (17), though its precursors have not been identified. No radiolabel was detected in fractions corresponding to lanosterol, desmosterol, or cholesterol upon incubation of 24(*S*),25-epoxy[^{3}H]lanosterol with IEC-6 cells. Thus, the metabolism of 24(*S*),25-epoxylanosterol in IEC-6 cells appears to be similar to that in rat liver (23).

The above observations support the hypothesis that the inhibition of HMG-CoA reductase activity observed upon treatment of IEC-6 cells with 24(*S*),25-epoxylanosterol may in fact be due to the formation of 24(*S*),25-epoxycholesterol as a result of cellular metabolism. The suppressive effect of 24(*S*),25-epoxycholesterol on reductase activity of cultured cells has been documented (16, 17). The inability of ketoconazole to completely protect reductase activity at higher concentrations of 24(*S*),25-epoxylanosterol (Fig. 5A), may be explained by the finding that the drug could not totally prevent the metabolism of 24(*S*),25-epoxylanosterol. Thus, the residual inhibition of HMG-CoA reductase activity may be attributed to small amounts of 24(*S*),25-epoxycholesterol (Fig. 6B) as well as other unidentified polar compounds (Fig. 7) that could still form in the presence of ketoconazole.

Effect of U18666A on the suppression of HMG-CoA reductase activity by oxylanosterols

We had previously demonstrated that the suppression of HMG-CoA reductase activity by SDO could be abolished by U18666A (14, 15). On the other hand, U18666A could not prevent the suppressive effect of a preformed oxysterol such as 25-hydroxycholesterol (15). From this indirect evidence, we had reasoned that the protective effect of U18666A was probably due to its interference with the cyclization of SDO. Since we could obtain the immediate cyclization product of SDO by chemical synthesis, it was of interest to verify our earlier hypothesis by studying the effect of U18666A on the suppression of HMG-CoA reductase activity by oxylanosterols. The results in **Fig. 8** demonstrate that U18666A was incapable of preventing the action of any of the oxylanosterols. On the other hand, the drug actually potentiated the inhibitory effect of 25-hydroxylanostene-3-one by nearly three-

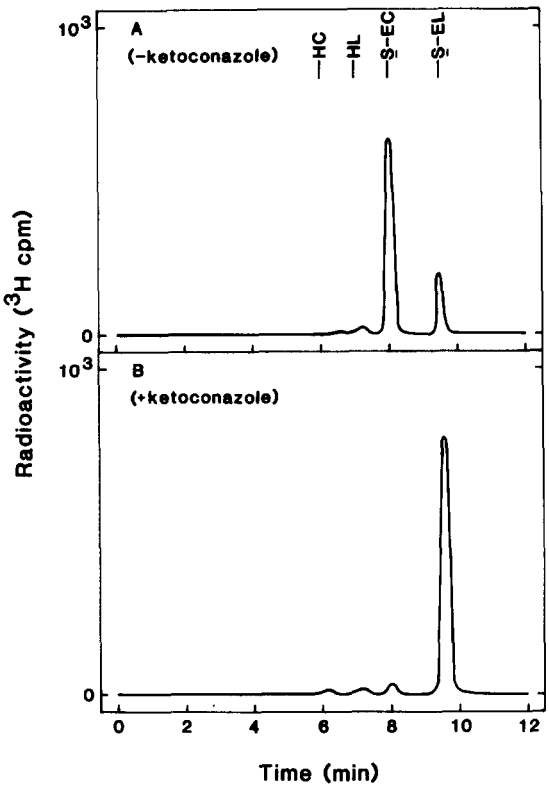

Fig. 7. Separation of metabolites of 24(*S*),25-epoxylanosterol by HPLC. IEC-6 cells were incubated for 6 hr with 24(*S*),25-epoxy[^{3}H]lanosterol (32,000 dpm) as described in the legend to Fig. 6. Nonsaponifiable lipid extracts from cells incubated with the radiolabel in the absence (A) or the presence (B) of 30 μM ketoconazole were separated by HPLC on a Zorbax ODS column (0.46 × 25 cm) in 100% methanol (1 ml/min). The column effluent was passed through a Flo-One model HP radioactivity detector. The output from the detector was integrated using a Gilson Data Master data analysis package (Full scale = 1000 cpm). The retention times of 25-hydroxycholesterol (HC), 25-hydroxylanosterol (HL), 24(*S*),25-epoxycholesterol (*S*-EC), and 24(*S*),25-epoxylanosterol (*S*-EL) are indicated.

fold (I_{50} values = 0.37 μM and 0.14 μM in the absence and presence of U18666A, respectively).

Effect of oxylanosterols on cellular cholesterol esterification

It is well documented that, in addition to suppressing HMG-CoA reductase activity of cultured cells, several oxysterols mimic low-density lipoproteins by enhancing the esterification of cellular cholesterol several-fold (36). This property is also exhibited by 24,25-epoxylanosterols and 25-hydroxylanosterol (**Table 3**). The extent of stimulation, though considerable, was less than that caused by 25-hydroxycholesterol. Surprisingly, 25-hydroxylanostene-3-one did not stimulate the esterification of cholesterol, even though it was a good suppressor of HMG-CoA reductase activity (Fig. 3).

DISCUSSION

Ever since Kandutsch, Chen, and Heiniger (3) proposed that oxysterols may be the intracellular modulators of sterol biosynthesis, the search has been on for the identification of such regulatory oxysterols whose endogenous production is not limited to a specific cell type. Oxygenated derivatives of lanosterol such as 32-hydroxylanosterol and 5α-lanostene-32-al, which are intermediates in the conversion of lanosterol to cholesterol, have been advanced as possible candidates for such a role (7, 37). These compounds were shown to inhibit sterol synthesis in cultured cells at the level of HMG-CoA reductase and also at the level of sterol 14α-demethylation (7, 37). It has been suggested (6) that under conditions of accelerated cholesterol synthesis from exogenous mevalonate (38, 39), the observed suppression of HMG-CoA reductase activity may in fact be due to intracellular accumulation of 32-oxygenated lanosterol derivatives. In recent years, a second group of natural compounds has emerged as strong candidates for a similar regulatory role. Studies from the laboratories of Spencer and Kandutsch (12, 13, 16, 17, 23) have indicated that side-chain epoxidized sterols are formed in human and animal tissues during the normal course of sterol synthetic activity. We have shown that, under conditions that cause partial inhibition of 2,3-oxidosqualene cyclase, squalene 2,3-epoxide could be di-

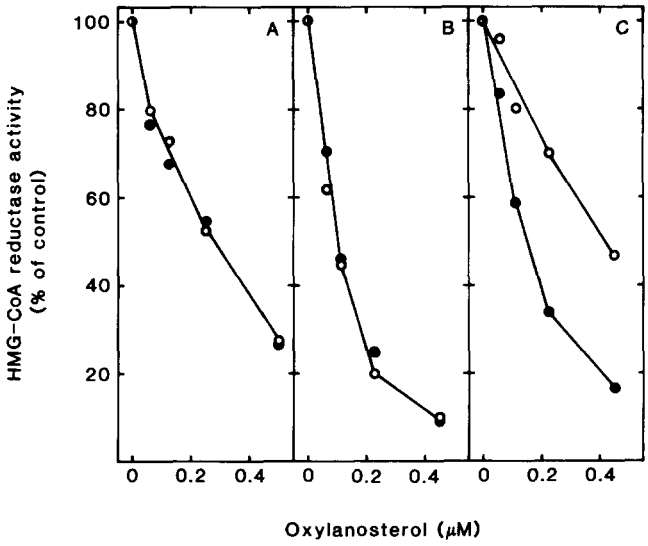

Fig. 8. Effect of U18666A on the suppression of HMG-CoA reductase activity in IEC-6 cells by oxylanosterols. Cells were pretreated for 1 hr with either no addition (○) or 2 μg/ml U18666A (●). Indicated concentrations of 24(*S*),25-epoxylanosterol (A), 25-hydroxylanosterol (B), or 25-hydroxylanostene-3-one (C) were then added. The activity of HMG-CoA reductase was determined in triplicate dishes at the end of 4 hr of incubation. One hundred percent values for enzyme activity (U/mg of protein) in the absence and presence of U18666A were 211 and 237, respectively.

TABLE 3. Effect of oxylanosterols on the esterification of cholesterol in IEC-6 cells

Oxysterol	Cholesterol Esterification (pmol/hr per mg of Protein)
None	158 ± 17
24(*S*),25-Epoxylanosterol	1,389 ± 89
24(*R*),25-Epoxylanosterol	1,104 ± 119
25-Hydroxylanosterol	1,544 ± 44
25-Hydroxylanostene-3-one	176 ± 19
25-Hydroxycholesterol	2,070 ± 107

Cholesterol esterification was determined by the incorporation of [^{3}H]oleic acid into cholesteryl esters by IEC-6 cell monolayers in the presence of the indicated oxysterols (2 μM) as described in the Experimental Procedures section. The results are expressed as the mean ± SEM of six determinations.

verted to side-chain oxygenated lanosterol derivatives via SDO in cultured cells (15). The results of the present study reveal that oxylanosterols are very potent inhibitors of sterol synthesis in cultured cells. Their site of inhibition appears to be localized at the step catalyzed by HMG-CoA reductase. A comparison of the patterns of inhibition of synthesis of C_{27}-sterols and of total nonsaponifiable lipids at oxylanosterol concentrations up to 2.3 μM revealed no evidence of inhibition of any post-mevalonate step. However, at relatively high concentrations (40 μM), 24(*S*),25-epoxylanosterol and 25-hydroxylanosterol have been reported to inhibit the conversion of lanosterol to cholesterol by rat liver homogenates (40).

In untreated IEC-6 cells, the level of 24(*S*),25-epoxylanosterol was extremely low. This was not unexpected since this compound is an intermediate in the formation of 24(*S*),25-epoxycholesterol. However, these cells could be induced to accumulate measurable amounts of the oxylanosterol through judicious use of specific inhibitors of the sterol biosynthetic pathway. Thus, by treating IEC-6 cells first with the oxidosqualene cyclase inhibitor, U18666A, and then with the 14α-demethylase inhibitor, ketoconazole, the endogenous concentration of 24(*S*),25-epoxylanosterol could be raised by >250-fold to a value of 500–550 fg/cell. In this context, the level of 24(*S*),25-epoxycholesterol in untreated Chinese hamster lung fibroblasts is reported to be 7.2 fg/cell (17).

While the above study demonstrates the ability of IEC-6 cells to synthesize 24(*S*),25-epoxylanosterol, the other two oxylanosterols, 25-hydroxylanosterol and 25-hydroxylanostenone, have not been shown to occur naturally. However, during the HPLC analysis of sterols from cells incubated with 24(*S*),25-epoxy[^{3}H]lanosterol, minor products were observed with retention times similar to 25-hydroxylanosterol and 25-hydroxycholesterol (Fig. 6). These compounds were generated in quantities insufficient for further characterization. It has been reported that Chinese hamster lung fibroblasts do synthesize small amounts of 25-hydroxycholesterol from radiolabeled mevalonate (16). The observation of Nelson, Steckbeck, and Spencer (12) that 25-hydroxylanosterol is efficiently converted to 25-hydroxycholesterol by rat liver homogenates raises the possibility that it could serve as a precursor to biosynthetic 25-hydroxycholesterol. Similarly, Tabacik, Aliau, and Sultan (24) recently found that human skin fibroblasts synthesize C_{30}-sterones from lanosterol such that they constitute a sizeable fraction of nonsaponifiable lipids generated from [^{14}C]acetate. Therefore, the regulatory significance of all the oxylanosterols documented in the current study warrants further investigation.

The data on the metabolism of 24(*S*),25-epoxylanosterol by IEC-6 cells in the presence and absence of cytochrome P_{450}-dependent C_{30}-sterol 14α-demethylase inhibitor, ketoconazole, support our earlier hypothesis (20) that the observed inhibition of HMG-CoA reductase activity and of sterol synthesis by 24(*S*),25-epoxylanosterol is dependent on its conversion to 24(*S*),25-epoxycholesterol. This finding is at variance with the results of Rachal et al. (19), who reported that exogenously added 24,25-epoxylanosterol did inhibit HMG-CoA reductase activity in a lanosterol demethylase-deficient mutant cell line. However, since these authors presumably used a racemic mixture of epoxylanosterols, it is possible that the 24(*R*),25-epoxylanosterol in the mixture was metabolized to 24(*R*)-hydroxylanosterol (23). Our observation that ketoconazole is much less effective in preventing the suppressive action of 24(*R*),25-epoxylanosterol (Fig. 5B) supports this possibility.

The finding that the 2,3-oxidosqualene cyclase inhibitor, U18666A, did not prevent the suppressive action of oxylanosterols (Fig. 8) confirms our earlier conclusion (13) that this drug blocks only the formation of endogenous oxysterols from SDO but not their subsequent action. The potentiation of the suppressive effect of 25-hydroxylanostene-3-one by U18666A is a finding that merits further study. It is possible that this could be due to enhanced uptake of the oxylanosterol in the presence of the drug. Alternatively, U18666A may have promoted the reduction of the 3-keto group of the sterone to a 3β-hydroxy group thus generating the more potent 25-hydroxylanosterol. In the absence of U18666A, such a reduction did not appear to take place since 25-hydroxylanostene-3-one did not stimulate cholesterol esterification in IEC-6 cells, while 25-hydroxylanosterol was an effective simulator of this activity (Table 3). The inability of 25-hydroxylanostene-3-one to enhance cellular cholesterol esterification suggests a possible role for a free 3β-hydroxy groups of sterols in stimulating acyl-CoA:cholesterol acyltransferase (ACAT) activity. Polyoxyethylated cholesterol, another sterol with a blocked 3β-hydroxy group, was similarly ineffective in stimulating cellular ACAT activity while being a potent suppressor of HMG-CoA reductase activity (41).

However, other reductase-suppressive steroids such as SC-31769, SC-31448, and SC-31082 contain a free 3β-hydroxy group but lack the ability to stimulate ACAT activity of cultured cells (42).

The data we have presented provide new evidence to support a role for oxylanosterols in the regulation of sterol synthesis and its major rate-limiting enzyme. Reports of natural occurrence of epoxysterols in biological tissues and cultured cells (13, 16, 17, 43, 44) add further emphasis to their regulatory role. In addition, preliminary studies appear to indicate that cholesterol may influence the accumulation of 24(*S*),25-epoxylanosterol (19, 43, 44). Thus, treatment of cultured cells with low density lipoproteins (19, 44) is reported to increase the cellular content of this epoxide. Similarly, feeding a cholesterol-rich meal to rats (43, 44) resulted in an accumulation of a sterol with the mass of 24(*S*),25-epoxylanosterol in the liver. Further experiments are under way to study the correlation between the rates of formation and cellular levels of such oxysterols and the regulation of HMG-CoA reductase in intact cells.

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