Oxygenation of desmosterol and cholesterol in cell cultures

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Abstract In order to determine whether hydration of the Δ^{24} bond of desmosterol contributes to the formation of the regulatory oxysterol, 25-hydroxycholesterol, [^{3}H]desmosterol was incubated with two cultured cell lines and the labeled products were analyzed. Small amounts of 25-hydroxycholesterol were formed with Chinese hamster lung (Dede) cell cultures, but not with mouse fibroblast (L) cell cultures. Apparently, desmosterol was converted into cholesterol, a process that does not occur in L cells, before 25-hydroxycholesterol takes place. No reliable evidence could be obtained for hydration of the Δ^{24} bond or for the reverse reaction upon incubation of [^{3}H]25-hydroxycholesterol. Oxygenation of desmosterol occurred in both Dede and L cell cultures to give a mixture of 24(*R*)- and 24(*S*)-25-epoxycholesterol. This reaction, along with the production of 7-oxygenated sterols, may account for low levels of HMG-CoA reductase repressor activity previously found to be associated with Δ^{24} sterols. —**Saucier, S. E., A. A. Kandutsch, A. K. Gayen, J. A. Nelson, and T. A. Spencer.** Oxygenation of desmosterol and cholesterol in cell cultures. *J. Lipid Res.* 1990. **31:** 2179–2185.

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25-Hydroxycholesterol is one of the most potent of the known sterol repressors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) reductase and this activity is correlated with its high affinity for the oxysterol receptor, a cytosolic protein that is thought to mediate oxysterol action (1). The detection of 25-hydroxycholesterol in Chinese hamster lung (Dede) cell cultures (2, 3) and in human (4) and mouse liver (5) suggests its possible role as a regulatory sterol. It is therefore of importance of know how 25-hydroxycholesterol arises in these biological media.

Enzymatic hydroxylation of cholesterol at C25, analogous to the well-documented enzymatic hydroxylations at C26 (6) and C24 (7, 8), is one obvious possibility for the origin of this oxysterol. It has been reported that enzymatic hydroxylation at the 25 position of cholesterol occurs to a minor extent in subcellular fractions of liver (8, 9). Biosynthesis of 25-hydroxycholesterol by way of squalene dioxide and 24(*S*),25-epoxycholesterol has also been suggested (10). However, no conversion of the latter compound to 25-hydroxycholesterol could be detected upon incubation of this epoxide with rat liver homogenates (11) or with cultured cells (12). It is known that 25-hydroxycholesterol can be produced by autoxidation of cholesterol (13), so the small amounts found in cells and tissues could originate in this way despite extensive efforts to prevent it. Some evidence that 25-hydroxycholesterol may be produced enzymatically as well as by autoxidation includes the detection of 25-hydroxycholesterol in untreated Dede cell cultures in the absence of the more common autoxidation products 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol (2). The absence of detectable levels of 25-hydroxycholesterol in some extracts of mouse liver (5) and in extracts of untreated Chinese hamster ovary (CHO) cell cultures grown in delipidated serum (S. E. Saucier and A. A. Kandutsch, unpublished results) indicates that autoxidative 25-hydroxylation of cholesterol is not inevitable in cell cultures or under the conditions used to isolate and analyze sterols.

The present investigation was sparked by the observation that weak HMG-CoA repressor activity was associated with 3β-hydroxy-Δ^{24} sterol fractions obtained from Dede cells and from liver (2, 3, 5). Since repressor activity was determined by incubating the fractions with L cell cultures, it seemed possible that this activity was caused by enzymatic hydration of the Δ^{24} bond to give 25-hydroxycholesterol in these cells. It also seemed conceivable that such enzymatic Δ^{24} hydration could be the major pathway for the formation of naturally occurring 25-hydroxycholesterol, a possibility that does not appear

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; HPLC, high performance liquid chromatography; desmosterol, cholest-5,24-dien-3β-ol; CHO, Chinese hamster ovary.

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to have been previously considered. Accordingly, it was decided to see whether the conversion of desmosterol to 25-hydroxycholesterol does occur in cultured cells.

EXPERIMENTAL PROCEDURES

Sterols

[1,2-^{3}H]Cholesterol (51.9 Ci/mmol) and 25-hydroxy [26,27-^{3}H]cholesterol (87 Ci/mmol) were obtained from New England Nuclear. [23-^{3}H]Desmosterol (14 Ci/mol) was synthesized as previously described (14). Before use, radioactive sterols were purified to a single homogeneous band with the retention time of the corresponding radioinert compound on a Resolve, 5 μm, C-18 HPLC column (Waters) using methanol–water 98:2 at a flow rate of 1 ml/min. 25-Hydroxycholesterol was obtained from Steraloids, Inc. and the synthesis of 24(*S*),25-epoxycholesterol, 24(*R*),25-epoxycholesterol, 24(*S*)-hydroxycholesterol (12), and 25-hydroxycholesterol monooleate (5) was as previously described.

Metabolism of sterols in cell cultures

Dede and L cells were grown as monolayers in Corning 150-cm^2 plastic culture flasks as described previously (2, 3), except that the concentration of vitamin E (*d,l*-α-tocopherol) added to the culture medium to inhibit autoxidation was increased to 5 μg/ml. Sterols were added to the cultures in a solution of ethanol and bovine serum albumin (12) and incubated at 37°C for 17 h. The cells were then scraped and washed twice with 0.14 M NaCl; an aliquot was taken for protein assay (15) and the remainder was pelleted. The average amounts of cellular protein per flask for Dede and L cell cultures were 9.4 ± 0.7 mg and 5.0 ± 0.5 mg, respectively. Lipids were extracted from the cells by the method of Bligh and Dyer (16) with the addition of butylated hydroxytoluene at a concentration of 1 μg/ml chloroform–methanol mixture to prevent autoxidation (2), the chloroform phase was dried under N_2, and the sterol fraction was separated from other lipids with a silica Sep-Pak (Waters) as described previously (2). An oxysterol fraction was separated from cholesterol by HPLC on a semi-preparative reverse phase C-18 column (Alltech), with methanol–hexane 90:10 which was changed to hexane at 69 min at a flow rate of 2 ml/min, and monitored for absorbance at 210 nm. This oxysterol fraction was resolved into a number of bands on a μPorasil column (Waters) with hexane–isopropyl alcohol 98.5:1.5 which was changed to hexane–isopropyl alcohol 97:3 at 22 min at a flow rate of 2 ml/min. The fractions corresponding to the retention times of cholest-5-en-3β,25-diol and 24,25-epoxycholest-5-en-3β-ol were then chromatographed on an analytical reverse phase Resolve column (Waters) with methanol–water 90:10 at 1 ml/min as described previously (2). Because the 7-oxygenated derivatives of desmosterol were not available for use as standards, the retention times of these compounds were calculated from those of the corresponding cholesterol derivatives by estimating the contribution of the Δ^{24} band as described previously (3). Fractions were collected and analyzed for ^{3}H. Calculated amounts (ng) of identified metabolites were based upon the specific activities of the administered sterol not taking into account possible dilution by endogenous sterols.

Chiral column HPLC

Stereochemical identification of the [23-^{3}H]24,25-epoxycholesterol formed in the incubations was accomplished on equipment previously described (5) by use of a 4.6 × 250 mm Chiralcel-OD column (J. T. Baker) with hexane–isopropyl alcohol 98:2 as the mobile phase at a flow rate of 0.5 ml/min. Two determinations were made which gave identical results. In the first, authentic samples of nonradioactive 24(*R*)- and 24(*S*),25-epoxycholesterol were added to the [23-^{3}H]24,25-epoxycholesterol and the peaks with the appropriate retention times were collected and counted. In the second, no radioinert standards were added.

RESULTS

In an initial study to determine whether 25-hydroxycholesterol might arise by hydration of the Δ^{24} bond of desmosterol, Dede and L cell cultures in 150-cm^2 flasks were incubated for 17 h with [23-^{3}H]desmosterol. Approximately 37% of the added desmosterol was taken up by the washed Dede cells and 35% by the L cells. As shown in **Table 1**, desmosterol was readily converted into cholesterol by Dede cells and, in addition, a small amount

TABLE 1. Compounds derived from [^{3}H]desmosterol in Dede and L cell cultures

^{3}H-Labeled Compounds with the Retention Times of the Following Sterols	Dede Cells	L Cells
	ng/mg protein	
Desmosterol[a]	6,323	20,420
25-Hydroxycholesterol[b]	1.5	n.d.[c]
7-Ketodesmosterol plus 7-ketocholesterol[d]	1.2	1.9
7(α and β)-Hydroxycholesterol plus 7(α and β)-hydroxy-desmosterol[d]	0.8	1.4
24,25-Epoxycholesterol[b]	1.0	2.1
Cholesterol[a]	3,701	n.d.

Dede and L cell cultures (two 150-cm^2 flasks) were incubated with [^{3}H]desmosterol, 0.88 μCi (26 μg)/ml, for 17 h. The pooled duplicate flasks were then assayed for protein, extracted, and the sterol mixtures were fractionated and assayed for ^{3}H.

[a]Purified through the semipreparative reverse phase HPLC step only.
[b]Purified through the analytical reverse phase (Resolve) HPLC step.
[c]n.d., Not detected (absence of a detectable radioactive peak).
[d]Purified through the normal phase (μPorasil) HPLC step.

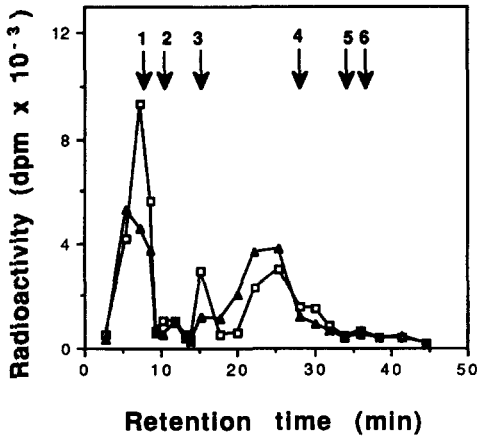

Fig. 1. Normal phase (μPorasil) HPLC of sterols produced from [^{3}H]desmosterol in Dede (□—□) and L (▲—▲) cell cultures. [^{3}H]Desmosterol, 0.88 μCi (26 μg)/ml, was incubated for 17 h with cultures grown in 150-cm^2 flasks. The oxysterols extracted from the cells were separated from desmosterol and other lipids by semipreparative, reverse phase HPLC and then the oxysterol band was rechromatographed on μPorasil. Arrows indicate retention times of standard 24,25-epoxycholesterol, 1; 24(*S*)-hydroxycholesterol, 2; 25-hydroxycholesterol, 3; 7-ketocholesterol, 4; 7β-hydroxcholesterol, 5; and 7α-hydroxycholesterol, 6.

of radioactivity cochromatographed with 25-hydroxycholesterol (**Fig. 1** and **Fig. 2**). Bands were also isolated which cochromatographed with 7-keto and 25-hydroxycholesterols and with 24,25-epoxycholesterol (Figs. 1 and 2). These latter results were also obtained with L cells (Table 1, Figs. 1 and 2), but the L cells, which lack the enzyme that catalyzes reduction of the Δ^{24} bond (17), did not convert desmosterol to cholesterol or, detectably, to 25-hydroxycholesterol.

If the formation of 25-hydroxycholesterol were indeed occurring by enzymatic hydration of desmosterol, it might be anticipated that the reaction would be detectably reversible. Hydration–dehydration reactions of alkenes have equilibrium constants close to unity, and there are several enzymatic examples for which reversibility can easily be demonstrated experimentally (18, 19). Accordingly, conversion of 25-hydroxy[^{3}H]cholesterol to desmosterol was tested with the results shown in **Table 2** and **Fig. 3** and **Fig. 4**. Approximately 19% of the added 25-hydroxycholesterol was taken up by the Dede cells and 39% by the L cells. A very low level of radioactivity cochromatographed with desmosterol, suggesting that dehydration to form a Δ^{24} double bond could have occurred to a very limited extent in both Dede and L cells. However, further investigations of the compound were not feasible because the level of radioactivity was too low. Even if the formation of [^{3}H]desmosterol could be established conclusively, it would remain to be demonstrated that the dehydration was enzymatic. Conversion of 25-hydroxycholesterol into small amounts of unidentified compounds more polar than 25-hydroxycholesterol (fractions 9 and 10 in Fig. 3) and to highly nonpolar compounds, one of which had the retention time of 25-hydroxycholesterol oleate (fractions 40 to 50 of Fig. 3), occurred in agreement with previous observations (20).

In order to gain further insight into the origin(s) of 25-hydroxycholesterol, [^{3}H]cholesterol was also incubated with Dede and L cells under the same conditions used with desmosterol and 25-hydroxycholesterol. Approximately 29% of the added cholesterol was taken up by the Dede cells and 8% by the L cells. As indicated in Tables 1 and 2 and in **Fig. 5** and **Fig. 6**, in Dede cell cultures similar amounts of [^{3}H]cholesterol and [^{3}H]desmosterol were converted into 25-hydroxy[^{3}H]cholesterol. The amount of 25-hydroxy[^{3}H]cholesterol was too small to quantitate by absorbancy measurement at 210 nm so specific activities could not be calculated, and the degree to which [^{3}H]cholesterol, either produced from [^{3}H]desmosterol or taken up from the medium, equilibrated with the endogenous cellular level of approximately 20 μg of

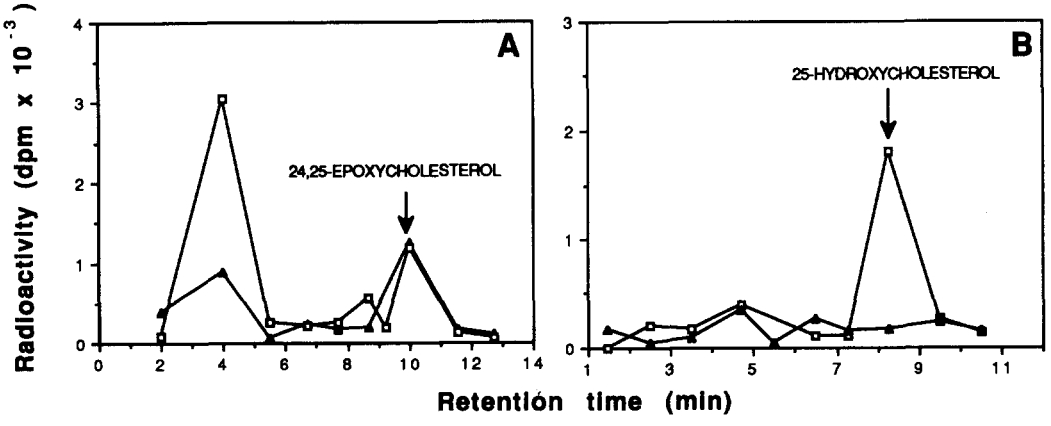

Fig. 2. Analytical, reverse phase rechromatography of radiolabeled fractions in Fig. 1 from Dede (□—□) and L (▲—▲) cell cultures with the retention times of 24,25-epoxycholesterol (A) and 25-hydroxycholesterol (B). Arrows indicate the retention times of the standard sterols.

TABLE 2. Compounds derived from 25-hydroxy[^{3}H]cholesterol and [^{3}H]cholesterol in Dede and L cell cultures

^{3}H-Labeled Compounds with the Retention Times of the Following Sterols	25-Hydroxy[26,27-^{3}H]cholesterol		[1,2-^{3}H]Cholesterol	
	Dede	L Cell	Dede	L Cell
	ng/mg protein			
Desmosterol	0.1[a]	0.2[a]		
25-Hydroxycholesterol	706[b]	2,839[b]	2.5,[c] 1.8[c]	n.d.,[a,d] n.d.[c]
7-Ketodesmosterol			0.4,[c] 5.3[c]	1.0,[a] 1.6[c]
7(α,β)Hydroxycholesterol			0.2,[c] 2.0[a]	0.8,[a] 0.9[a]
24,25-Epoxycholesterol			n.d.,[c] n.d.[a]	n.d.,[a] n.d.[a]
Cholesterol	n.d.[a]	n.d.[a]	6,150,[b] 9,343[b]	2,880,[b] 2,126[b]

Cultures were incubated with [^{3}H]cholesterol, 0.88 μCi (26 μg)/ml or [^{3}H]25-hydroxycholesterol, 0.8 μCi (5 μg)/ml. Protein and sterols were analyzed as in Table 1.

[a]Purified through the normal phase (μPorasil) HPLC step.

[b]Purified through the semipreparative, reverse phase HPLC step.

[c]Purified through the analytical reverse phase (Resolve) HPLC step.

[d]n.d., Not detected (absence of a detectable radioactive peak).

radioinert cholesterol/mg protein before 25-hydroxylation occurred could not be determined. To the extent that equilibration did occur, the values shown for Dede cells in Tables 1 and 2 could be larger by as much as fourfold. Significant, but variable, oxygenation at the 7-position also occurred. As expected, since oxidative conversion of cholesterol to 24,25-epoxycholesterol is chemically implausible, no peak of radioactivity with the retention time of this epoxide was found.

The results obtained when [^{3}H]cholesterol was incubated with L cell cultures were similar to those obtained with Dede cells except that production of 25-hydroxy-[^{3}H]cholesterol was undetected in L cells. Since desmosterol is the major endogenous sterol of L cells, there was no dilution of the labeled cholesterol in these cells and the mass of 25-hydroxycholesterol produced in Dede cells as compared to L cells may therefore be several-fold greater than is indicated by these values.

Further evidence for the identity of the radiolabeled compound that cochromatographed with 25-hydroxycholesterol was obtained by recrystallizing with authentic 25-hydroxycholesterol the radioactive material that had been formed from both desmosterol and cholesterol and eluted from the reverse phase analytical column. As shown in **Table 3**, the specific activity of the radioactive sterol produced in Dede cells from [^{3}H]desmosterol and from [^{3}H]cholesterol was not significantly changed after five recrystallations.

The identity of the ^{3}H-labeled compound cochromatographing with 24,25-epoxycholesterol was further established by chiral column chromatography of the putative

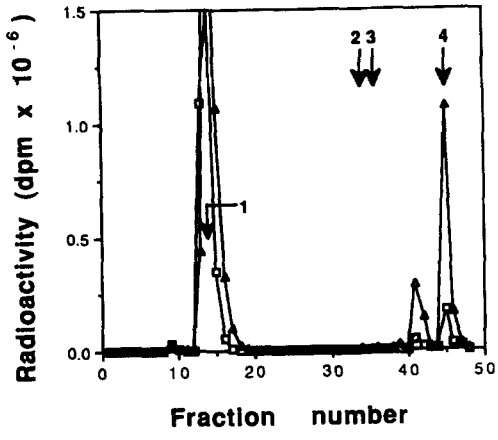

Fig. 3. Semipreparative, reverse phase HPLC of extracts from Dede (□—□) and L (▲—▲) cell cultures incubated with 25-hydroxy[^{3}H]-cholesterol. 25-Hydroxy[^{3}H]cholesterol, 0.8 μCi (5 μg)/ml, was incubated for 17 h with the cultures and the extracted lipids were chromatographed. Volumes collected for fractions 1-33 and 36-48 were 1 ml and 6 ml, respectively. Arrows indicate the retention times of standard 25-hydroxycholesterol, 1; desmosterol, 2; and cholesterol, 3; 25-hydroxycholesterol monooleate, 4.

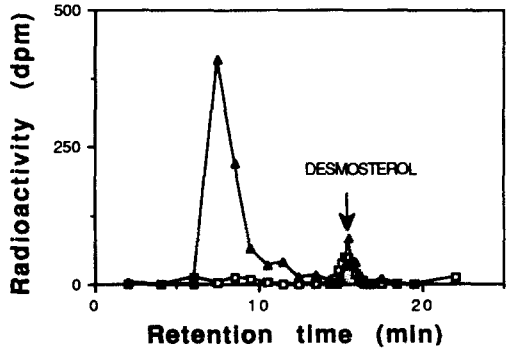

Fig. 4. Normal phase (μPorasil) chromatography of radiolabeled fraction 34 in Fig. 3 from Dede (□—□) and L (▲—▲) cell cultures with the retention time of desmosterol. The sterols were eluted with hexane-isopropyl alcohol 95.5:0.5 pumped at a flow rate of 2 ml/min. The arrow indicates the retention time of the standard sterol.

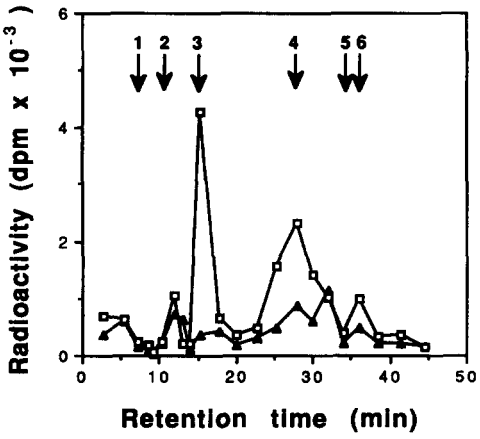

Fig. 5. Normal phase (μPorasil) HPLC of sterols produced from [^{3}H]cholesterol in Dede (□—□) and L (▲—▲) cell cultures. [^{3}H]Cholesterol, 0.88 μCi (26 μg)/ml, was incubated for 17 h with cultures grown in 150-cm^2 flasks. The sterol mixtures extracted from the cells were fractionated by semipreparative, reverse phase HPLC and the radioactive oxysterol band was then rechromatographed on μPorasil as described in Fig. 1. Arrows indicate retention times of standard sterols: 24,25-epoxycholesterol, 1; 24(*S*)-hydroxycholesterol, 2; 25-hydroxycholesterol, 3; 7-ketocholesterol, 4; 7β-hydroxycholesterol, 5; 7α-hydroxycholesterol, 6.

epoxide from L cell cultures. Material from L cell cultures was chosen for this experiment because these cells do not metabolize either 24(*S*),25-epoxycholesterol or 24(*R*),25-epoxycholesterol, whereas Dede cells convert 24(R),25-epoxycholesterol to 24(*R*)-hydroxycholesterol (12). By the procedure described in Materials and Methods it was determined that 46.4% of the 24,25-epoxycholesterol was the 24(*S*) epimer (retention time: 63.8 min) and 53.6% was the 24(*R*) epimer (retention time 70.5 min).

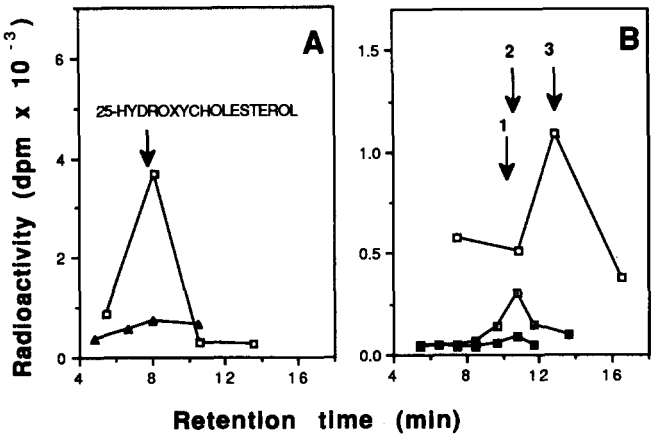

Fig. 6. Analytical, reverse phase rechromatography of fractions in Fig. 5. A: Fraction 10 from Dede (□—□) and L (▲—▲) cell cultures with retention time corresponding to 25-hydroxycholesterol. B: Fractions 15, 18, and 19 from Dede cells corresponding to the retention times of 7-ketocholesterol, 7β-hydroxycholesterol, and 7α-hydroxycholesterol were rechromatographed. The arrows indicate the retention times of standard 7α-hydroxycholesterol, 1; 7β-hydroxycholesterol, 2; and 7-ketocholesterol, 3.

TABLE 3. Recrystallization with authentic sterol of putative 25-hydroxy[^{3}H]cholesterol produced in Dede cells from [^{3}H]desmosterol or [^{3}H]cholesterol

	Putative 25-Hydroxy[^{3}H]cholesterol	
Crystallization	From [23-^{3}H]Desmosterol	From [1,2-^{3}H]Cholesterol
	dpm/mg	
0	57.7	378
1	60.2	362
2	68.8	365
3	66.0	350
4	60.6	369
5	64.2	361

Radiolabeled bands similar to those illustrated in Figs. 2B and 6A were dried, mixed with 30 mg of authentic 25-hydroxycholesterol, and crystallized. Approximately 1 mg of the dried crystals was weighed and analyzed for ^{3}H.

DISCUSSION

Since the amounts of 25-hydroxycholesterol formed in Dede cells from desmosterol and cholesterol are comparable, hydration of the Δ^{24} double bond of desmosterol does not appear to be an important source of 25-hydroxycholesterol relative to hydroxylation of cholesterol. Hydration of desmosterol to give 25-hydroxycholesterol was not detected in L cells and could not be determined in Dede cells because in this cell line desmosterol is rapidly reduced to cholesterol, which was separately shown to be subject to 25-hydroxylation. Evidence that the reverse dehydration reaction may have occurred to an extremely small extent was inconclusive.

It is still uncertain whether the 25-hydroxylation of cholesterol is in part or entirely enzymatic. However the virtual absence of this reaction in L cell cultures and the absence of any quantitative correlation between the generation of 25-hydroxycholesterol and the more common autoxidation products, 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol, argue for enzymatic catalysis of the reaction. Our previous observations that 25-hydroxycholesterol is present in cultured Dede cells (2, 3), but not in CHO cells grown under similar conditions (S. E. Saucier and A. A. Kandutsch, unpublished data), provide further circumstantial support for enzymatic 25-hydroxylation since the probability of autoxidation of endogenous cholesterol should be the same in both cases.

If enzymatic 25-hydroxylation of cholesterol occurs in some cell cultures, then the addition of cholesterol to the medium could result in repression of HMG-CoA reductase. However, even in the unlikely case that the [^{3}H]cholesterol taken up by Dede cells was completely equilibrated with endogenous cholesterol, the amount of 25-hydroxycholesterol produced in 17 h (approximately 4 × 2 = 8 ng/mg protein) was only approximately equal

to the previously determined endogenous level of 6–11 ng/mg protein (2, 3). Whereas an increase of this magnitude would be expected to have a detectable effect upon HMG-CoA reductase activity in Dede cells (2), the amounts produced in a shorter incubation period of 5 h, regularly used in our assays for oxysterol repression of HMG-CoA reductase activity, would not be sufficient to have any significant effect (21, 22).

The generation of 24,25-epoxycholesterol from desmosterol under the conditions of these experiments was unexpected. Metabolic production of 24(*S*),25-epoxycholesterol from squalene dioxide occurs in cell cultures and in liver, but enzymatic epoxidation of the sterol Δ^{24} bond has not been reported. However, slow autoxidation of desmosterol in air to give, presumably, a mixture of the 24 *R* and *S* epimers of 24,25-epoxycholesterol has been reported (23, 24). Since approximately equal amounts of the two epimers were produced in L cell cultures under the conditions of our experiments, it appears most likely that they were generated by a nonenzymatic mechanism. Such generation of 24,25-epoxycholesterol, along with 7-oxygenated sterols, may account for some or all of the weak reductase repressor activity found when high concentrations of Δ^{24} sterols are incubated with L cell cultures.

These results illustrate the difficulties that can arise in attempting to analyze the causes of weak reductase repressor activity associated with a sterol or sterol mixture. Since total suppression of all autoxidation reactions cannot be ascertained, the only conclusive evidence for enzymatic catalysis may be chiral specificity of the product. On the other hand, formation of a mixture of epimers may not in itself be evidence against catalysis by an uncharacterized enzyme. The results described herein also demonstrate that inefficient reactions of the kind examined here, whether enzymatic or autoxidative, may occur differentially in different cell types and should be anticipated under the usual conditions requisite for cell viability.

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