Metabolism of 25-hydroxycholesterol in mammalian cell cultures. Side-chain scission to pregnenolone in mouse L929 fibroblasts

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Abstract Three mammalian cell lines were examined for their ability to metabolize the regulatory oxysterol, 25-hydroxycholesterol, and derepress 3-hydroxy-3-methylglutaryl CoA reductase. In mouse L cell fibroblasts reductase activity was restored with the concomitant metabolism of 25-hydroxycholesterol via side-chain hydroxylation and scission of the C20–C22 bond. Chinese hamster lung cells did not appear to derepress the reductase and these cells and Chinese hamster ovary cells did not metabolize 25-hydroxycholesterol to a significant extent. Only 5–10% of the oxysterol became esterified with a fatty acid in any of the cell lines when grown in the described culture conditions.—**Taylor, F. R., and A. A. Kandutsch.** Metabolism of 25-hydroxycholesterol in mammalian cell cultures. Side-chain scission to pregnenolone in mouse L929 fibroblasts. *J. Lipid Res.* 1989. **30:** 899–905.

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The addition of a polar substituent, such as a keto or hydroxyl function, at certain positions will transform cholesterol and lanosterol into potent repressors of cholesterol biosynthesis in cultured mammalian cells (1, 2). One of the most active of these compounds, 25-hydroxycholesterol, has been shown to specifically reduce the levels of certain cholesterogenic enzymes (3, 4) by repressing the transcription of their genes (5, 6); it has also been shown to increase the rate of degradation of 3-hydroxy-3- methylglutaryl CoA reductase (HMGR) (7, 8). These discoveries support the proposal that cholesterol biosynthesis in cells is normally regulated by endogenously synthesized oxysterols (1). Several natural oxysterols have been isolated from the tissues and serum of mice and humans, and from cells in culture. These include 32-hydroxylanosterol and 32-oxolanosterol, which are formed as enzymatic intermediates in the 32-demethylation of lanosterol (9), 24S, 25-epoxycholesterol, which is formed as a side product of the cholesterol biosynthetic pathway after the introduction of an additional epoxide function into 2,3-epoxysqualene (10, 11), and derivatives of cholesterol hydroxylated at the 24, 25, or 26 positions in the side chain, presumably catalyzed by cytochrome P450 enzymes (11, 12, 13). Increases in oxysterol concentrations are correlated with repression of HMGR when cells are treated with mevalonate (9), inhibitors of oxidosqualene cyclase (14) or lanosterol 14-demethylase (15), or when mice are fed cholesterol (13).

In order for oxysterols to act as regulators of cholesterol biosynthesis there must be mechanisms to reduce their levels in cells under conditions where an increase in cholesterol biosynthesis is required. Possible mechanisms include decreased synthesis, increased efflux, or metabolism to an inactive or less active form. Mechanisms for deactivating oxysterols are suggested by an analysis of the oxysterol structural features that are required for potent repressor activity. These features include a free 3β-hydroxyl group and a full-length side chain as well as the additional polar substituent (16). Thus, the level of a regulatory oxysterol could be diminished by further metabolism, i.e., 14-demethylation of 32-oxylanosterol intermediates, cleavage of the side chain, or esterification of the 3β-hydroxyl with either a fatty acid or sulfate ester.

The metabolism in mammalian cells of regulatory oxysterols with an oxygen function in the side chain has not been thoroughly investigated. Erikson et al. (17) found that 25-hydroxycholesterol was rapidly converted to more polar compounds in rat liver, although these metabolites were not identified. Side-chain hydroxylated sterols, including 25-hydroxycholesterol, have been shown to undergo side-chain scission in endocrine cells (18). Unlike the side-chain scission of cholesterol, metabolism of hydroxylated sterols does not require stimulation by trophic hormones (18); these more water-soluble sterols are thought

Abbreviations: HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; pregnenolone, 3β-hydroxy-5-pregnen-20-one.

to by-pass the step that regulates the movement of cholesterol to the site of P450scc-catalyzed scission in the inner mitochondrial membrane. We have previously investigated the metabolism of 24,25-epoxy derivatives of both cholesterol and lanosterol during short-term incubations with cell cultures (19). The lanosterol derivatives were rapidly converted to the epoxycholesterol analogues and the unnatural *R* enantiomer of the 24,25-epoxide was found to undergo reduction to the 24-hydroxyl group. Further, less rapid metabolism of these compounds was also evident in these experiments.

In this report we describe studies on the metabolism of 25-hydroxycholesterol in three cell lines. L cell cultures metabolized added 25-hydroxycholesterol to noninhibitory products and correlated derepression of HMGR occurred. In these cells 25-hydroxycholesterol underwent side-chain hydroxylation and scission to give 3β-hydroxy-5-pregnen-20-one (pregnenolone). Little metabolism of 25-hydroxycholesterol was found in Chinese hamster lung and ovary cells, and esterification did not seem to be an important metabolic route in any of the cell lines under the culture conditions used in these studies.

MATERIALS AND METHODS

Materials

Waymouth's MB752/1 medium was from Hazleton Research Products; McCoy's 5A medium was from KC Biological; and fetal bovine serum was from Gibco. Culture flasks were from Corning. 25-Hydroxy[26, 27-^{3}H]cholesterol, 25-hydroxy[16,17-^{3}H]cholesterol, [1,2-^{3}H]cholesterol, and [7-^{3}H]pregnenolone were from New England Nuclear. Unlabeled cholesterol and 25-hydroxycholesterol were from Steraloids, and pregnenolone was from Mann Research; all three were recrystallized before use. The 3β-oleate ester of 25-hydroxycholesterol was synthesized in this laboratory by Sandra Saucier (13). Tetrabutylammonium phosphate was from Kodak and bovine serum albumin and α-tocopherol were from Sigma. High performance liquid chromatography (HPLC) solvents were from Burdick and Jackson.

Cell lines and culture media

Mouse L cell fibroblasts (a subline of NCTC clone 929) were grown in spinner culture in serum-free Waymouth's MB752/1 medium modified as described (20). Chinese hamster lung (Dede) and ovary (CHO) cells were grown in culture flasks in McCoy's 5a medium supplemented with 4 mg/ml of delipidated (21) fetal bovine serum proteins.

Incubation of cells with 25-hydroxycholesterol and extraction of metabolites

Cells (2–3 × 10^6) from the L cell spinner culture or from trypsinized Dede or CHO cell cultures were pipetted into a 25 cm^2 culture flask. After the cells had attached, the medium was poured off and replaced with 2–3 ml of fresh medium without serum protein. Radiolabeled 25-hydroxycholesterol was added to the medium in 0.2 ml of a solution of 5% bovine serum albumin and 1% ethanol with 5 μg/ml α-tocopherol as an antioxidant. After incubation for various periods at 37°C in a 5% CO_2 atmosphere, the cells were suspended in the medium with a rubber policeman and collected by centrifugation, washed once in 2 ml of 0.14 M NaCl and then resuspended in 1 ml of 0.14 M NaCl. Aliquots of the medium and washed cells were analyzed for radioactivity to determine oxysterol uptake. Sterols in the cells and the medium were extracted by adding equal volumes of acetone and allowing the mixtures to stand at 4°C overnight. Precipitated cellular debris was removed by centrifugation, one volume of chloroform was mixed with the supernatant fluid, and the resulting solvent phases were separated by centrifugation.

Chromatography

For thin-layer chromatography (TLC), aliquots of the chloroform–acetone and water–acetone phases were applied directly to the pre-adsorbant strip of Whatman LK6 silica gel 60A thin-layer plates. The chloroform–acetone extracts were developed with toluene–ethyl acetate 67:33 and the water–acetone extracts were developed with chloroform–methanol–water 60:40:4. Fractions of the plates were scraped and counted in Tritosol scintillation solvent (22). Where indicated, tetrabutylammonium phosphate was added to the TLC developing solvents to a final concentration of 0.3 g/100 ml (9 mM). For HPLC, aliquots of the chloroform–acetone phase were dried under nitrogen, resuspended in methanol–water 75:25 and analyzed on a reversed phase "Resolve" 5 μm C18 column (Waters) using as eluant methanol–water 75:25 at a flow rate of 1 ml/min. Fractions were collected and counted in Tritosol. Recovery of counts from the medium and cells after extraction and chromatography averaged over 90%.

Other procedures

Total cell protein was determined by sonicating the washed cell suspensions and assaying them by the procedure of Lowry et al. (23). HMGR activity was determined as described (16). Mass spectrum analysis was performed on a Hewlett-Packard 5985B gas chromatograph–mass spectrometer at the mass spectrometry facility in the Department of Chemistry, University of Maine, Orono, using electron impact mode (2800 EMV).

RESULTS

The loss of 25-hydroxycholesterol repressor activity during incubation with cell cultures: correlation with its metabolism

The metabolic fate of 25-hydroxycholesterol was examined in L, CHO, and Dede cell lines, which are routinely used in this laboratory for the study of oxysterol regulation of cholesterol biosynthesis. Incubation of these cells with 25-hydroxycholesterol results in the rapid repression of HMGR activity. In L cell cultures a concentration of 170 nM 25-hydroxycholesterol represses enzyme activity by 50% after 5 h of incubation (16), while Dede and CHO cultures are more refractory and require somewhat higher oxysterol concentrations. To test whether these cell lines can inactivate physiologically relevant concentrations of oxysterols and derepress HMGR activity, the cultures were incubated with 250 nM 25-hydroxycholesterol and HMGR activity was monitored over the next 50 h. As expected, HMGR activity was repressed by ~55% in L cells after 5 h of incubation (**Fig. 1**, top panel). After 15 h HMGR activity began to recover and by 40 h it increased to nearly twice the 5 h level. Because the medium contains a large reservoir of 25-hydroxycholesterol which must be depleted to derepress HMGR activity, it was necessary to seed the cultures at high cell density to see significant recovery. Regulatory oxysterols produced by intracellular metabolism might be expected to undergo more rapid depletion. The initial decline in HMGR activity in the untreated control cultures was probably due to the nutritional depletion of the fresh medium provided at the beginning of the experiment (24). HMGR activity in Dede cultures was not inhibited by 25-hydroxycholesterol to as great an extent as in L cells, and recovery from this inhibition was not apparent (Fig. 1, bottom panel). These results suggested the possibility that L cells, but not Dede cells, had the capability to inactivate regulatory oxysterols.

In parallel with the measurement of HMGR activity, the extent of oxysterol metabolism in cultures incubated with 25-hydroxy[26, 27-^{3}H]cholesterol was monitored by silica gel TLC analysis of extracts of the cells and medium. In L cell cultures the amount of radiolabel with the same R_f as 25-hydroxycholesterol declined throughout the experiment in both the cells (**Fig. 2A**) and the medium (Fig. 2B) with less than 15% remaining after 51 h. This decline was accompanied at first by an increase in more polar radioactive material in the chloroform phase of the medium extract (Fig. 2B). At later time points an increased percentage of the radioactivity was found in the water–acetone phase of the medium extract (Fig. 2B). This material showed a single band on TLC. These observations suggested that the initial polar product(s) were undergoing further metabolism to a more polar compound. In addition, at early time points, 4–5% of the cell-associated radioactivity chromatographed with an R_f in the same region as 25-hydroxycholesteryl-3β-oleate ester and this percentage increased to 10.8% by the 51 h time point. Further identification of this nonpolar material was not attempted. These results for the kinetics of HMGR derepression and oxysterol metabolism in L cells are representative of two replicate experiments.

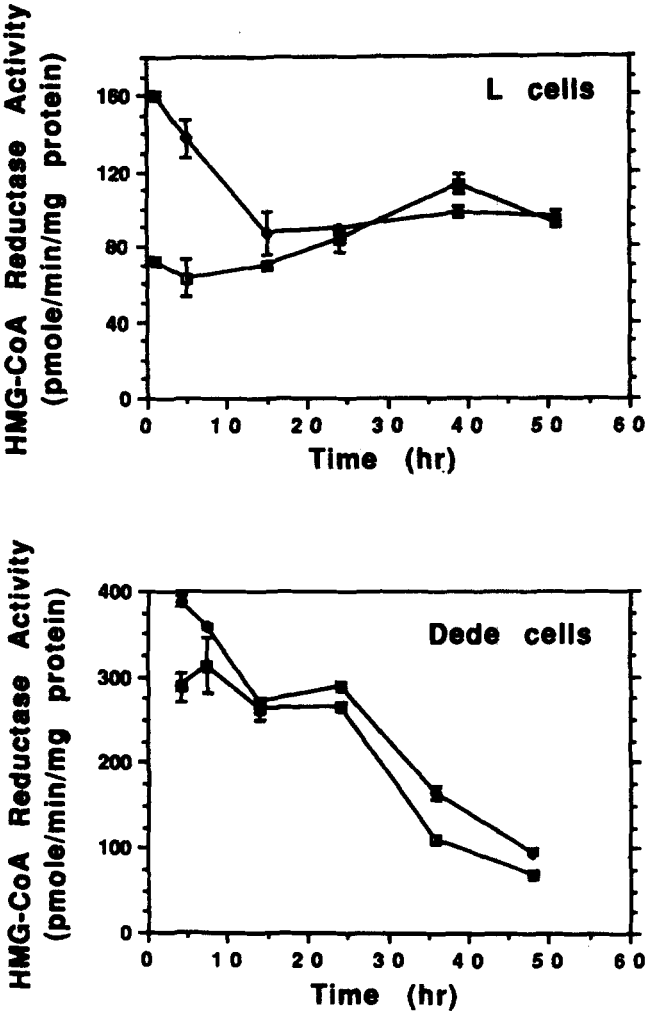

Fig. 1. Derepression of HMGR activity during incubation with 25-hydroxycholesterol. L cells (top panel) and Dede cells (bottom panel) were treated (-□-), starting at zero time, with 250 nM 25-hydroxycholesterol. Control cultures (-■-) were incubated without oxysterol. Culture flasks were harvested at the indicated times and cell homogenates were assayed for HMGR activity. The error bars indicated the range of the mean in duplicate assays; when the error bars are not visible the error was less than the size of the symbol.

In Dede cells a much smaller percentage of the 25-hydroxycholesterol was converted to other metabolic products. After 60 h of incubation 82–85% of the cell-associated radioactivity remained unmetabolized, 7–10% chromatographed on silica gel as the fatty acid ester of 25-hydroxycholesterol, and 7–11% of the material was more polar than 25-hydroxycholesterol (**Fig. 3A**). The chloroform extract of the Dede culture medium contained

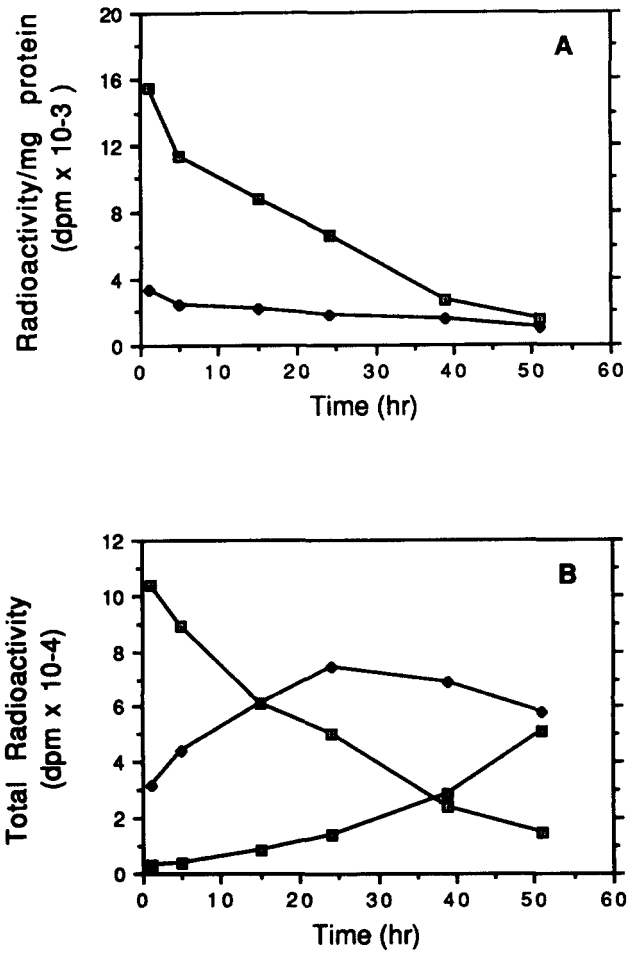

Fig. 2. Metabolism of 25-hydroxy[26,27-^{3}H]cholesterol in L cells. L cell cultures were incubated, starting at zero time, with 250 nM tritiated 25-hydroxycholesterol (0.5 μCi/ml). Culture flasks were harvested at the indicated time points and the washed cell pellets (panel A) and the medium (panel B) were extracted and analyzed on silica gel TLC. Symbols: (-□-), the 25-hydroxycholesterol fraction from the chloroform phase of the extracts; (-◆-) more polar sterols in the chloroform phase of the extracts; and (-■-) radioactivity in the aqueous phase of the medium extract.

mostly (78%) unmetabolized 25-hydroxycholesterol with a higher percentage (21%) of more polar products than found in the cell extract (Fig. 3B). The water-acetone phase of the cell and medium extracts contained negligible radioactivity. CHO cells gave very similar results (data not shown). In control experiments 25-hydroxycholesterol incubated in medium without cells but with the antioxidant α-tocopherol and then extracted and analyzed by silica gel TLC yielded ~10% autoxidized polar sterols. Differences between these cell lines in the extent of repression of HMGR and the rate of metabolism of 25-hydroxycholesterol did not appear to be due to differences in the uptake of oxysterol into the cells. The amount of added 25-hydroxycholesterol found associated with the washed cell pellet after 3 h of incubation was 11% in L cells and 8–10% in CHO and Dede cells, and this percentage increased to 15–17% in all cell lines at 15 h.

Identification of the metabolic products of 25-hydroxycholesterol

A clue to the metabolic events that deactivate 25-hydroxycholesterol in L cells came from a comparison of the metabolic products formed from this oxysterol labeled with tritium at either the 16, 17 or 26, 27 positions. Both sterols gave rise to polar material in the chloroform phase of the medium extracts which remained near the origin on silica gel TLC (**Fig. 4A and 4B**). The side chain-labeled sterol also yielded radioactivity in the water-acetone phase of the medium extract (Fig. 2B). This product was not formed in incubations with the ring-labeled sterol and instead a new radiolabeled band was found in the chloroform phase which migrated as a compound less polar than 25-hydroxycholesterol on silica gel TLC (Fig. 4A). These

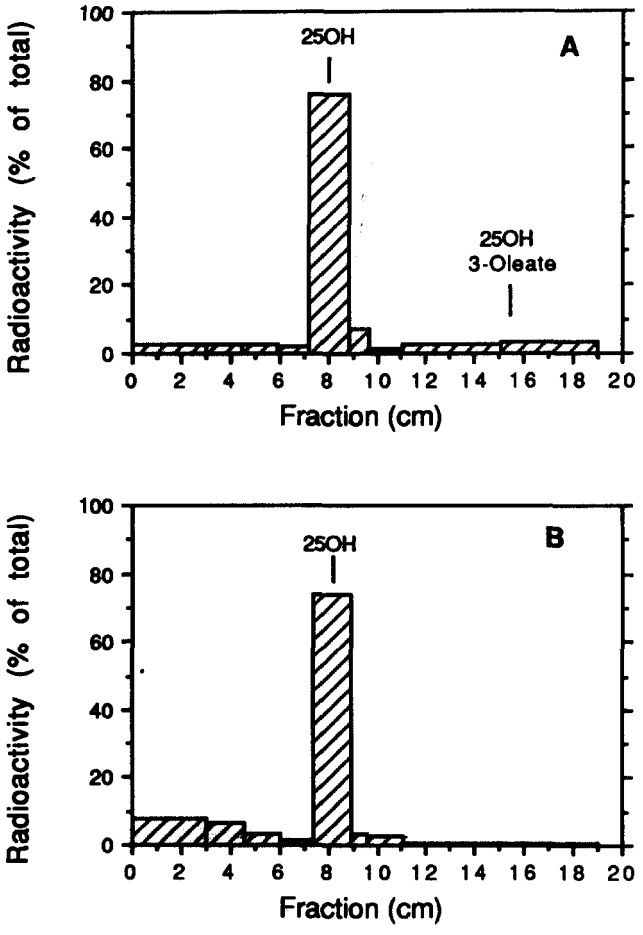

Fig. 3. Metabolism of 25-hydroxy[16,17-^{3}H]cholesterol in Dede cells. Dede cells were incubated with 250 nM tritiated 25-hydroxycholesterol (0.5 μCi/ml) for 60 h. The washed cell pellet (panel A) and the medium (panel B) were extracted and the chloroform phase of the extracts was analyzed on silica gel TLC.

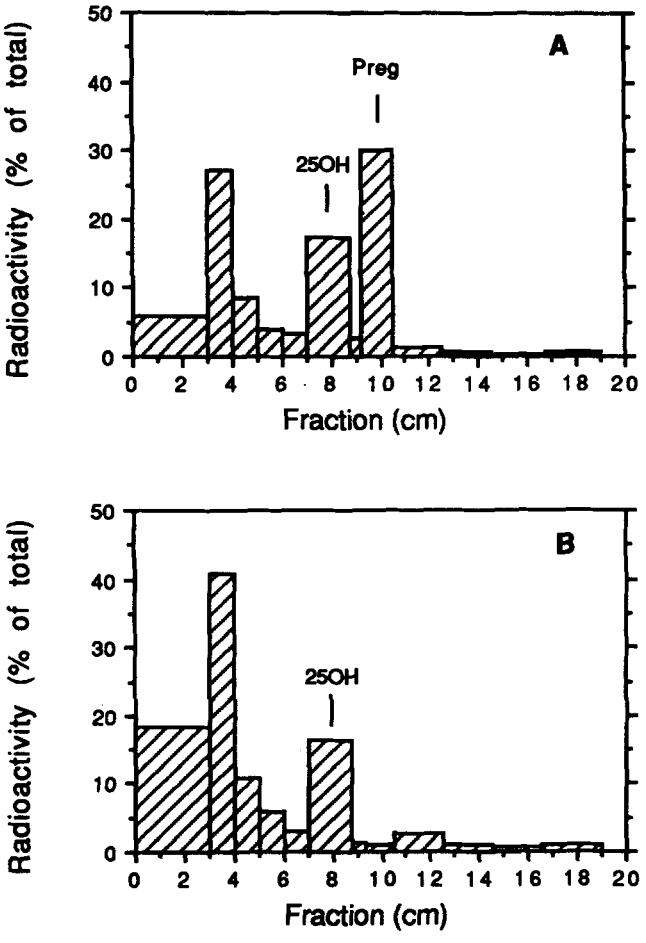

Fig. 4. Metabolism of tritiated 25-hydroxycholesterol in L cells. L cell cultures were incubated with 250 nM of tritiated (0.5 μCi/ml) of 25-hydroxycholesterol labeled at C16, C17 (panel A) or C26, C27 (panel B) for 60 h. The chloroform phase of the extract of the medium was analyzed on silica gel TLC.

results suggested that the water-soluble tritiated fragment from the side chain-labeled sterol was being released by side-chain scission. If scission was between the C20–C22 bond, the expected products would be pregnenolone and 4-hydroxy-4-methylpentanaldehyde. This scission of the side chain was confirmed by identification of the radioactive product, which was slightly less polar than 25-hydroxycholesterol on silica gel TLC, as pregnenolone. This product co-migrated with pregnenolone on silica gel TLC (Fig. 4A) and on a reverse phase C18 HPLC column (data not shown). The radioactive band was mixed with 50 mg of pregnenolone to give a specific activity of 657 dpm/mg and recrystallized from ethanol–water: first recrystallization, 598 dpm/mg; second recrystallization, 644 dpm/mg; third recrystallization, 654 dpm/mg; fourth recrystallization, 629 dpm/mg.

The time course of metabolite production shown in Fig. 2 B suggested that the polar metabolite(s) near the origin of the silica gel chromatogram might be a precursor in the production of pregnenolone, namely the C20- or C22-hydroxylated or C20, 22-dihydroxylated intermediates of side-chain cleavage. Resolution of this more polar radioactive band by reverse-phase HPLC revealed two components in a 3 to 1 ratio with the more polar of the two being the major product (data not shown). The more polar band was oxidized with periodic acid by incubating an aliquot estimated to contain 4 nmol of the polar metabolite with 34 nmol of sodium periodate in 10 μl of methanol–water 1:1. After standing 36 h at room temperature the solution was analyzed by silica gel TLC. Most (85%) of the product of the oxidation reaction co-chromatographed with pregnenolone, indicating the presence of adjacent 20,22-hydroxyl functions in this metabolite. Mass spectroscopy of a small quantity of the metabolite did not yield the expected mass ion at *m/z* 434. However, fragmentation products were found at *m/z* (ion formula intensity relative to base peak at *m/z* 43 = 100) 401 (M^+- CH_3–H_2O, 0.7), 360 (M^+–CH_3–C_3H_7O, 1.2), 317 (M^+–C_6 $H_{13}O_2$, 26.8) and 299 (M^+–$C_6H_{13}O_2$–H_2O, 19.9), the last two products arising from cleavage at the C20, C22-diol. This cleavage has been reported as a characteristic product in the mass spectrum of trimethylsilated (20R, 22R) 20,22- dihydroxycholesterol (25).

The identity of the small percentage of polar products derived from 25-hydroxycholesterol in Dede and CHO cell cultures was not established. This material was much more polar on reversed phase HPLC than the putative 20, 22-dihydroxy derivative of 25-hydroxycholesterol produced by L cells, eluting immediately after the void volume on a C18 column developed with methanol–water 3:1 (v/v). Its mobility on silica gel was not affected by tetrabutyl ammonium phosphate in the developing solvent. Since this counterion increases the mobility of cholesteryl sulfate on silica gel, this result suggests that the polar material was not sulfated.

DISCUSSION

The potent inhibitory effect of oxysterols on cholesterol biosynthesis and the demonstrated presence of these sterols, including 25-hydroxycholesterol, in tissues, serum, and cell lines led us to begin an examination of the ability of cells to regulate the intracellular levels of oxysterols by metabolic transformation to inactive products. Mouse L cell fibroblast cultures proved to have the capability of metabolizing 25-hydroxycholesterol by hydroxylating the C20 and C22 positions of the side chain followed by scission of a portion of the C20,22-dihydroxylated product to pregnenolone. Moreover, the time course of this metabolism was correlated with the derepression of HMGR activity. These results were surprising because the L cell fibroblast line was de-

from normal subcutaneous areolar and adipose tissue (26) and it was not expected to have steroidogenic enzymes; incubation of L cells with [^{3}H]pregnenolone did not yield any further metabolic transformations (data not shown) indicating that the steroidogenic pathway is not complete. Furthermore, in endocrine tissues the C20, C22, and C20,22 hydroxylated intermediates in the side-chain scission of cholesterol do not accumulate to an appreciable extent (27), whereas in our experiments the C20,22 dihydroxylated derivative of 25-hydroxycholesterol was the major metabolite found. A possible explanation for the accumulation of this intermediate may be that the high concentration of 25-hydroxycholesterol available to the side-chain scission system in these cell cultures displaces the hydroxylated intermediate, which then is not retained by the cells and is less available for further metabolism.

Incubation of L cells with [^{3}H]mevalonic acid and with [^{3}H]cholesterol in the presence or absence of 1 mM 8-bromoadenosine 3':5'-cyclic monophosphate, which induces the side-chain scission of cholesterol in endocrine cells, did not yield radiolabeled pregnenolone (data not shown) suggesting that the side-chain scission pathway in this cell line is accessible only to oxysterols. To our knowledge this is the first report of side-chain scission activity in a nonendocrine tissue or in a cell line derived from a nonendocrine tissue. It remains to be determined whether this activity in L cells is due to abnormal gene activation of the endocrine P450$_{scc}$ in this cell line, perhaps due to an advantage gained by inactivating oxysterols present in serum-containing medium, or to the expression of a natural capability of the somatic cells from which this line was derived.

The Dede and CHO cells did not express side-chain hydroxylation and scission activity and had little ability to metabolize 25-hydroxycholesterol to esters or to other deactivated products. This was reflected in the apparent inability of Dede cells to recover HMGR activity after treatment with low doses of 25-hydroxycholesterol. Although 25-hydroxycholesterol stimulates the esterification of cholesterol in some mammalian cells (28), esterification of oxysterols with long-chain fatty acids appears not to be a major route for deactivating exogenously supplied oxysterols in any of the three cell lines when grown in the lipid-free culture conditions used in this investigation.

The results presented here and in previous publications document that the metabolism of oxysterols in cultured cells can affect their potency as repressors of HMGR. Reduction of 3-keto sterols to 3β-hydroxy derivatives (16), the metabolism of oxylanosterols to cholesterol derivatives (19), and the reduction of 24(R), 25-epoxycholesterol to 24R-hydroxycholesterol (19) all serve to increase the apparent potency of those sterols. In the present case further oxidation of the oxysterol side chain results in decreased retention of the oxysterol by the cells, and subsequent cleavage to pregnenolone deactivates the sterol completely because an intact side chain is required for repressor activity. Thus, measurements of the potency and kinetics of oxysterol repression of HMGR may be influenced in several ways by metabolic transformation of the sterols during the course of the assay.

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