Effects of specific inhibition of sterol biosynthesis on the uptake and utilization of low density lipoprotein cholesterol by HepG2 cells

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Abstract Treatment of HepG2 cells in lipoprotein-deficient media with 4,4,10β-trimethyl-*trans*-decal-3β-ol (TMD) abolished the incorporation of [^{3}H]acetate into cholesterol with concomitant accumulation of squalene 2,3(*S*)-oxide and squalene 2,3(*S*):22(*S*),23-dioxide, indicating a specific inhibition of oxidosqualene cyclase. The activity of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase was affected in a biphasic manner, being inhibited by 30% at low concentrations of TMD and stimulated by 30% at concentrations that completely shut down oxidosqualene cyclase. Treatment with TMD (>20 μg/ml) doubled the specific binding and internalization of low density lipoproteins (LDL) and also enhanced their degradation to a degree comparable to that produced by lovastatin, a well-known inhibitor of HMG-CoA reductase. The enhanced binding of LDL to HepG2 cells appeared to occur as a result of an increase in the number of binding sites with no change in their binding affinity for the lipoprotein. At concentrations that completely inhibited cholesterol biosynthesis, TMD did not affect the ability of LDL-derived cholesterol to stimulate cholesterol esterification by seven- to tenfold or to stimulate bile acid secretion to a lesser degree. However, TMD treatment inhibited overall bile acid secretion by 75–85%. The compound had no inhibitory effect on the rates of secretion of either apolipoprotein B or of cholesterol by HepG2 cells into the culture medium. These data demonstrate that a specific inhibition of the sterol branch of isoprenoid biosynthetic pathway in hepatic cells by TMD is sufficient to induce the expression of LDL receptors and that the cholesterol delivered by LDL is available for normal metabolic purposes of the cell. —**Panini, S. R., G. T. Everson, and T. A. Spencer.** Effects of specific inhibition of sterol biosynthesis on the uptake and utilization of low density lipoprotein cholesterol by HepG2 cells. *J. Lipid Res.* 1991. **32:** 1657–1665.

Supplementary key words LDL receptors • bile acid secretion • apolipoprotein B • cholesterol esterification • HMG-CoA reductase

Cholesterol in human plasma is transported complexed mainly to low density lipoproteins (LDL). The uptake of cholesterol from the blood by various tissues is mediated by specific receptors on the surface of cells in the liver and extrahepatic tissues (1). The expression of such receptors is stringently regulated as part of the maintenance of cellular cholesterol homeostasis.

Inasmuch as the expression of LDL receptors is presumably controlled by cellular levels of cholesterol (2), it logically follows that agents that inhibit cholesterol biosynthesis would be expected to induce receptor levels. Indeed, such is the case upon treatment of animals or cultured cells with lovastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol synthesis (3, 4). However, the site of inhibition by lovastatin in the branched pathway of isoprenoid biosynthesis is a step common to the production of cholesterol as well as of several nonsterol prenyl derivatives (5). Therefore, an examination of the role of biosynthetic sterols in the regulation of lipoprotein receptor levels requires a specific inhibition of the sterol branch of the pathway. Induction of LDL receptor activity of cultured cells by ketoconazole, an inhibitor of cytochrome P_{450}-mediated reactions, has been previously reported (6–8). However, since ketoconazole can affect several steps involved in the biosynthesis and catabolism of cholesterol, its use has not allowed an accurate assessment of the specific role of biosynthetic sterols in the regulation of LDL receptor expression.

While the plasma membranes of most cells have LDL receptors, liver accounts for more than half of the total receptors in the body and is the organ responsible for irreversible removal of cholesterol from the blood (9). Increasing numbers of investigations have focussed on the

Abbreviations: LDL, low density lipoproteins; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TMD, 4,4,10β-trimethyl-*trans*-decal-3β-ol; MEM, Eagle's minimal essential medium; LPDS, lipoprotein-deficient serum; HPLC, high performance liquid chromatography; apoB, apolipoprotein B; ACAT, acyl coenzyme A:cholesterol acyltransferase.

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regulation of hepatic lipoprotein receptors. Due to the considerations of complexity, cost, and time involved in the study of liver metabolism in intact animals, cultured hepatocytes and established hepatoma cell lines are becoming model systems of choice. In this context, the human hepatoblastoma-derived cell line, HepG2, has been shown to express regulable LDL receptors (10, 11) as well as to exhibit several aspects of lipid metabolism comparable to that of normal human liver (12). HepG2 cells are capable of bile acid synthesis and secretion (13) and secrete lipoproteins of very low density (VLDL), low density (LDL), and high density (HDL) into the culture medium (14, 15).

In the current study, we set out to examine the effects of specific inhibition of cholesterol biosynthesis on the uptake and utilization of LDL cholesterol by HepG2 cells. Our choice for the inhibitor was 4,4,10β-trimethyl-*trans*-decal-3β-ol (TMD), a known specific inhibitor of 2,3-oxidosqualene cyclase in rat liver homogenates (16) and in cultured Chinese hamster ovary (CHO) cells (17). We have previously shown that TMD treatment does not interfere with nonsterol regulation of HMG-CoA reductase (18). In addition, unlike several late stage inhibitors of cholesterol synthesis that are nonspecific cytochrome P_{450} inhibitors (19), TMD also does not interfere with the regulatory actions of LDL-derived cholesterol in cultured cells (20). We have compared the results with TMD in HepG2 cells to those with lovastatin to test the hypothesis that the expression of LDL receptors is regulated entirely by sterols. We have also examined the effects of TMD on the utilization of cholesterol by these cells for such varied purposes as bile acid secretion, lipoprotein secretion, esterification, and regulation of HMG-CoA reductase.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]acetic acid (22 Ci/mmol) and carrier-free sodium [^{125}I]iodide (17 Ci/mg) were purchased from ICN Biomedicals, Costa Mesa, CA. [4-^{14}C]cholesterol (54 mCi/mmol) was obtained from Research Products International, Mount Prospect, IL. [9, 10-^{3}H](n)-oleic acid (13 Ci/mmol), *R,S*-[3-^{14}C]HMG-CoA (58 mCi/mmol), and *R,S*-[^{5}H]mevalonolactone (27 Ci/mmol) were from Du Pont New England Nuclear, Boston, MA. TMD was prepared as described previously (21). Lovastatin was a generous gift from Merck, Sharpe and Dohme, Rahway, NJ and was converted to the sodium salt prior to use. Low density lipoprotein (LDL, $1.02 < d < 1.063$ g/ml) was prepared from fresh human plasma by ultracentrifugation using KBr for density adjustment (22). All other chemicals were of analytical grade.

Cell culture

HepG2 cells (catalog #HB 8065) were obtained from American Type Culture Collection, Rockville, MD. Cells were routinely grown at 37°C in a humidified 5% CO_2 atmosphere in Eagle's Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum. For the experiments, cells were grown to confluence in 35-, 60-, or 100-mm diameter culture dishes in growth medium and then re-fed MEM supplemented with 10% lipoprotein-deficient serum (LPDS). The LPDS ($d > 1.21$ g/ml) was prepared from new-born calf serum by ultracentrifugation after adjusting the density to 1.21 g/ml with KBr (22). After dialysis against normal saline, the LPDS was diluted to a protein concentration of 40 mg/ml, to match that of fetal calf serum, and filter-sterilized using a 0.2-μm filter.

HPLC analysis of sterols

Incorporation of radiolabel into newly synthesized sterols was determined as described previously for Chinese hamster ovary cells (18). Briefly, cells in LPDS medium were incubated with [^{3}H]acetate (5 μCi/ml) for 6 h. Monolayers were washed with saline and total lipids were extracted into hexane–isopropyl alcohol 60:40 as described previously (22, 23). Lipids were saponified in 1 N KOH for 30 min at 75°C in methyl alcohol–benzene 80:20. Nonsaponifiable lipids were extracted into hexane for separation by reverse phase high performance liquid chromatography (HPLC) on a 4.1 × 300 mm Versapak C_{18} column (Alltech Associates, Deerfield, IL) using acetonitrile–water 90:10 as the mobile phase at 2 ml/min. The column effluent was monitored by an on-line radioactivity detector (Radiomatic, Tampa, FL).

Metabolism of ^{125}I-labeled LDL

Iodination of LDL and the determination of specific binding and internalization were carried out essentially as described by Goldstein, Basu, and Brown (22). Degradation of LDL to monoiodotyrosine by HepG2 cells was determined as described by Drevon et al. (24).

Analytical methods

The activity of HMG-CoA reductase was assayed in detergent extracts of cells as described previously (25), with the modification that the assay time was limited to 30 min. Bile acids were extracted from the culture medium and quantified by a sensitive isotope dilution gas chromatography mass spectrometry technique as described previously (13). ^{14}C-labeled bile acids synthesized from added [^{14}C]cholesterol were isolated from the culture medium using Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) as described before (13), and were resolved from minor amounts of conjugated bile acids and

from cholesterol (26) by thin-layer chromatography on silica gel GHL glass-backed plates (Analtech, Newark, DE) developed in isopropyl alcohol–isooctane–dioxane–acetic acid 7:10:6:2. Total and free cholesterol were determined by an enzymatic method (27) in equivalent aliquots of hexane–isopropyl alcohol 60:40 extracts of cells. The difference between the two values was taken to represent esterified cholesterol. For the measurement of secretion of apolipoprotein B (apoB) by HepG2 cells, serum-free culture medium from each 100-mm culture dish was concentrated tenfold using an Amicon YM-30 membrane and the levels of apoB were determined in multiple aliquots by electroimmunoassay (28) using an anti-human LDL antiserum (Sigma, St. Louis, MO). For the measurement of cholesterol secretion, culture media from parallel incubations were centrifuged at 12,000 *g* for 20 min and the supernatant fractions were extracted according to Bligh and Dyer (29). Total cholesterol content of the chloroform phase was then determined as above (27). The rate of esterification of cellular cholesterol was determined using [^{3}H]oleic acid complexed to bovine serum albumin as described previously (22). Statistical significance was assessed by Student's *t*-test.

RESULTS

Inhibition of sterol biosynthesis in HepG2 cells by TMD

Treatment of HepG2 cells with TMD for 24 h caused a dose-dependent inhibition of cholesterol synthesis from [^{3}H]acetate with concomitant accumulation of squalene epoxides (**Fig. 1**). It should be pointed out that while the distribution of radioactivity among individual components was altered, incorporation of radiolabel into total nonsaponifiable lipid fraction was essentially unchanged at all concentrations of TMD tested. At TMD concentrations $\geq$20 μg/ml, analysis of radiolabeled nonsaponifiable lipids by reverse phase HPLC revealed the accumulation of only squalene 2,3(*S*)-epoxide and squalene 2,3(*S*):22(*S*),23-dioxide at the expense of cholesterol. At an intermediate concentration of TMD (10 μg/ml), formation of small amounts of cholesterol and 24(*S*),25-epoxycholesterol, the two end products of squalene epoxide metabolism, could be detected. The specificity of TMD effect on sterol biosynthesis is confirmed by the fact that the compound did not affect the synthesis of total fatty acids from [^{3}H]acetate. Incorporation of radioactivity (dpm $\times$ 10^{-6} per mg protein) into total fatty acids was 1.13 $\pm$ 0.04, 1.14 $\pm$ 0.05, 1.17 $\pm$ 0.01, and 1.16 $\pm$ 0.10 for control, 10 μg/ml, 20 μg/ml, and 40 μg/ml TMD, respectively. Measurement of the activity of HMG-CoA reductase under these conditions revealed a minor ($\approx$30%) but significant ($P < 0.05$) decrease at a TMD concentration of 10 μg/ml and a minor ($\approx$30%) stimulation ($P < 0.05$) at a TMD concentration of 40 μg/ml (**Fig. 2**).

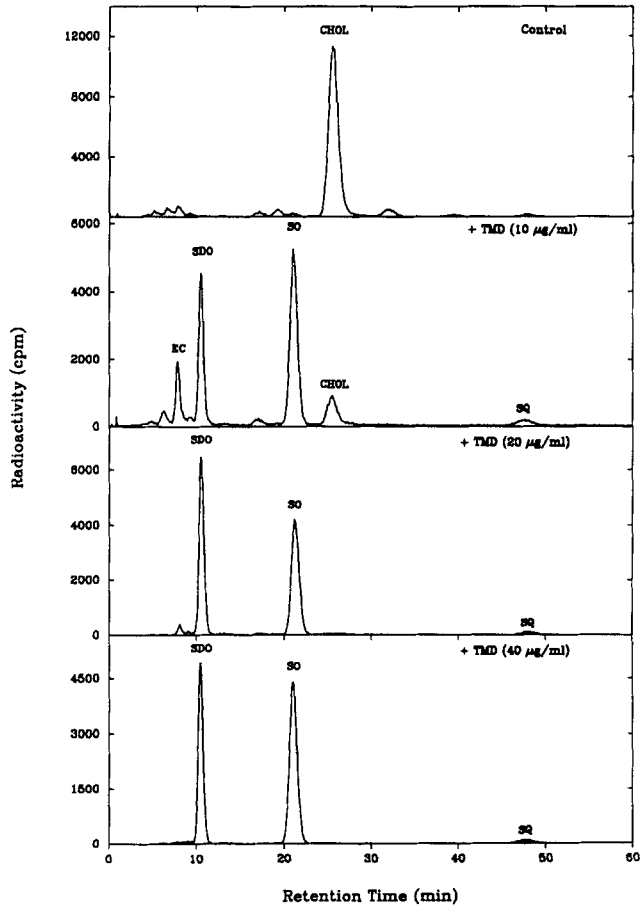

Fig. 1. Effect of TMD on the biosynthesis of cholesterol by HepG2 cells. Confluent monolayers of HepG2 cells in 35-mm culture dishes were rinsed twice with Puck's Saline G and re-fed MEM supplemented with 10% lipoprotein-deficient serum (high density fraction of serum, d > 1.215 g/ml, LPDS) containing indicated concentrations of TMD. After 18 h incubation, cells were pulsed for 6 h with [^{3}H]acetate (5 μCi/ml). Cellular lipids were extracted and nonsaponifiable lipids were analyzed on a Versapak C_{18} reverse phase HPLC column using acetonitrile–water 90:10 as the mobile phase (2 ml/min) as described under Experimental Procedures. The effluent from the column was monitored for radioactivity by an on-line detector. A representative trace of radioactivity detector is shown for each treatment. Biosynthetic cholesterol (CHOL), 24(*S*),25-epoxycholesterol (EC), squalene (SQ), squalene 2,3-epoxide (SO), and squalene 2,3:22,23-dioxide (SDO) were identified by coelution of authentic mass standards at respective retention times.

We also assessed possible toxic effects of TMD on HepG2 cells by monitoring the rates of incorporation of [^{35}S]methionine into total cellular proteins after exposure of cells to 20 and 40 μg/ml TMD for 24 and 48 h in the presence or absence of 100 μg/ml LDL. No adverse effects of TMD on the rates of protein synthesis were observed (data not shown).

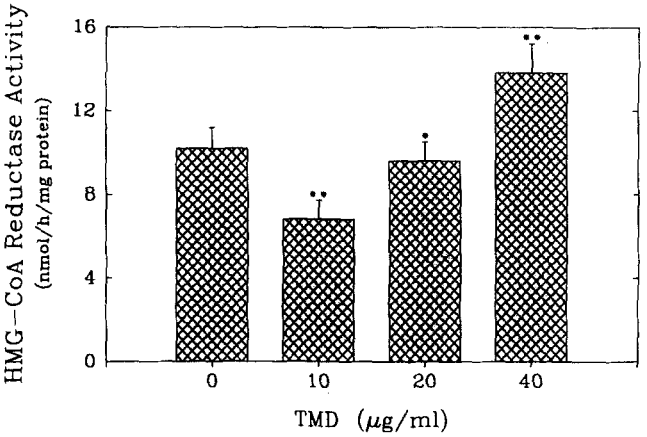

Fig. 2. Effect of TMD on the activity of HMG-CoA reductase in HepG2 cells. HepG2 cells in 35-mm culture wells were preincubated for 24 h in LPDS medium. They were then re-fed fresh LPDS medium containing indicated amounts of TMD. After 24 h incubation, triplicate dishes were harvested for the determination of HMG-CoA reductase activity as described under the Experimental Procedures. The data are presented as the mean ±SEM of results from two experiments (n = 6). $^{*}P > 0.1$ versus zero TMD control; $^{**}P < 0.05$ versus zero TMD control.

Effect of TMD on ^{125}I-labeled LDL metabolism

Treatment of HepG2 cells with concentrations of TMD that caused complete inhibition of sterol biosynthesis resulted in two- to threefold enhancement of specific binding and internalization of ^{125}I-labeled LDL (**Table 1**). Specific degradation was also increased, but to a lesser degree. The magnitude of these increases was comparable to that produced by a dose of lovastatin (6 μg/ml) sufficient to cause cessation of all isoprenoid synthesis. Scatchard analysis of LDL binding data (**Fig. 3**) indicated that TMD treatment of HepG2 cells caused a dose-dependent increase in the number of LDL receptors with no change in binding affinity. The values for B_{max} (ng of LDL protein bound/mg of cell protein) were 160, 240, and 300, and for K_m (μg of LDL protein/ml) were 30.5, 30.6, and 29.1 in HepG2 cells treated with 0, 20, and 40 μg/ml TMD, respectively.

Effect of TMD on cholesterol utilization

Incubation of HepG2 cells in lipoprotein-deficient medium for 24 h with LDL (100 μg protein/ml) increased the total cholesterol content of cells, mainly through an enhancement in the amount of cholesteryl esters (**Table 2**). On the other hand, treatment with TMD lowered the total cholesterol content of the cells mainly through a decrease in the mass of free cholesterol. Incubation with both TMD and LDL resulted in cellular levels of total cholesterol similar to those seen after incubation with LDL alone. Thus, despite an increase in LDL receptor activity, TMD-treated cells did not accumulate greater amounts of total cholesterol, indicating that the incoming cholesterol was processed for export. The data in Table 2 indicate a 27% decrease ($P < 0.005$) in the mass of cellular cholesteryl esters in cells treated with both TMD and LDL when compared to cells treated with LDL alone. This decrease was not observed at a lower dose (20 μg/ml) of TMD (data not shown).

TABLE 1. Effect of inhibitors of cholesterol synthesis on LDL receptor activity of HepG2 cells

	^{125}I-Labeled LDL Metabolism		
	Binding	Internalization	Degradation
	ng/mg protein		
Control	57 ± 3	374 ± 9	701 ± 38
TMD (20 μg/ml)	130 ± 11[a]	891 ± 20[a]	990 ± 71[b]
Lovastatin (6 μg/ml)	106 ± 5[a]	856 ± 36[a]	927 ± 65[b]

Confluent HepG2 cells in 35-mm culture wells were incubated in LPDS medium for 24 h to induce the LDL receptors and for a further 24 h in LPDS medium supplemented with either TMD (20 μg/ml) or with lovastatin (6 μg/ml). Monolayers were then washed with Puck's saline G and incubated with fresh LPDS medium containing ^{125}I-labeled LDL (20 μg/ml) ± unlabeled LDL (1 mg/ml) for 6 h as described under Experimental Procedures. The data are the average ± SEM of triplicate cultures (n = 6).

[a]Different from corresponding control, $P < 0.001$.
[b]Different from control, $P < 0.01$.

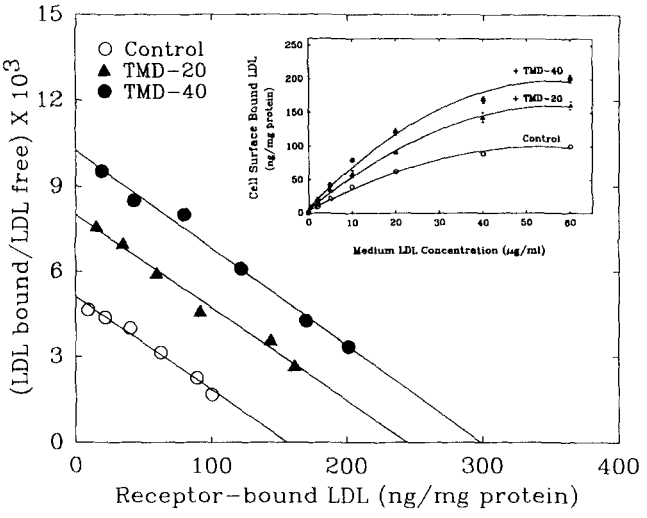

Fig. 3. Kinetics of binding of LDL to surface receptors of HepG2 cells. HepG2 cells were grown to confluence in 35-mm multi-well culture dishes in MEM supplemented with 10% fetal calf serum. Monolayers were rinsed twice with Saline G and were re-fed LPDS medium. The next day, cells were given fresh LPDS medium supplemented with either none (Control), 20 μg/ml (TMD-20), or 40 μg/ml (TMD-40) of TMD. After 24 h, monolayers were given 1 ml of LPDS medium containing 2-60 μg/ml of ^{125}I-labeled LDL ± 50-fold excess unlabeled LDL. After 6 h at 37°C, specific binding of LDL was determined as described under Experimental Procedures. The figure represents a recalculated Scatchard plot of the actual binding data obtained from triplicate determinations in duplicate experiments (n = 6) and shown in the inset. Recalculated data were fitted by least squares regression analysis.

TABLE 2. Effect of TMD on the cellular content of free and esterified cholesterol in HepG2 cells in the presence or the absence of LDL

Treatment	Cellular Cholesterol		
	Free	Ester	Total
	μg/mg protein		
Control	30.7 ± 1.1	7.9 ± 0.6	38.5 ± 0.9
LDL	32.0 ± 0.3	18.9 ± 0.6[a]	50.9 ± 0.8[a]
TMD	26.7 ± 0.3[a]	6.9 ± 0.3	33.6 ± 0.1[a]
TMD + LDL	39.2 ± 1.4[a, b, c]	13.9 ± 0.6[a, b, c]	53.1 ± 1.9[a, c]

Confluent HepG2 cells in 60-mm culture dishes were incubated for 24 h in LPDS medium and then re-fed fresh LPDS medium ± TMD (40 μg/ml). After 1 h, indicated dishes received LDL (100 μg protein/ml). Cholesterol content of the cells were determined after 24 h of treatment as described under Experimental Procedures. The results are the average ± SEM of triplicate cultures (n = 6).

[a] $P < 0.01$ versus control values in the same column.

[b] $P < 0.01$ versus values for LDL-treated cells in the same column.

[c] $P < 0.005$ versus values for TMD-treated cells in the same column.

Since HepG2 cells are capable of synthesizing and secreting bile acids, we examined the effect of incubation with TMD in the presence or absence of LDL on the rate of bile acid secretion by these cells. The basal rate of bile acid secretion by these cells varied widely from one experiment to the next. Nevertheless, the response of the secretion rate to supplementation of culture media with TMD and/or LDL was relatively constant. The data presented in **Table 3** are from a representative experiment. Supplementation with LDL stimulated total bile acid secretion of HepG2 cells by 22%. Incubation with TMD inhibited the rate of secretion by 85%, thus supporting the contention that newly synthesized cholesterol is the preferred substrate for bile acid biosynthesis (9, 30, 31). Addition of LDL to TMD-treated cells partially overcame the inhibition. It is interesting to note that the mass increase in bile acid secretion rate in the presence of LDL was similar in both control (12.5 ng/h per mg protein) and TMD-treated (11 ng/h per mg protein) cells. Taken together, these data suggest that LDL-derived cholesterol is unable to sustain normal rates of bile acid synthesis in TMD-treated HepG2 cells. This could be due to either a direct inhibition of bile acid biosynthetic pathway by TMD or a lack of availability of LDL-derived cholesterol to microsomal cholesterol 7α-hydroxylase, the first and rate-limiting enzyme in bile acid biosynthesis.

To test the possibility of a direct inhibition of bile acid biosynthesis by TMD, we examined the effect of the compound (20 and 40 μg/ml) on the conversion of exogenous [^{14}C]cholesterol to water-soluble bile acid products in the culture medium by HepG2 cells. TMD did not appear to affect such conversion at either concentration (data not shown). Analysis of alkali digests of cells revealed negligible amounts of bile acids, indicating that TMD did not cause a sequestration of bile acids inside the cells. Due to the extremely low rates of enzyme activity exhibited by HepG2 microsomes (32), it was not possible to ascertain whether TMD treatment had an inhibitory effect on the cholesterol 7α-hydroxylase activity of these cells.

The possibility that TMD might have interfered with the intracellular trafficking of LDL-derived cholesterol was investigated next. It was reasoned that such an interference might affect pathways in addition to that of bile acid biosynthesis. In HepG2 cells, free cholesterol may be directed into multiple pools that also serve as substrates for esterification, regulatory sterol formation, and lipoprotein assembly (32, 33).

As pointed out above, the data in Table 2 suggested that TMD, at 40 μg/ml, may have affected re-esterification of cholesterol derived from exogenous LDL. We examined this process directly by measuring esterification of cellular cholesterol by exogenous [^{3}H]oleic acid. As shown in **Fig. 4**, HepG2 cells incubated in LPDS medium have a low basal rate of cholesterol esterification known to be catalyzed by acyl coenzyme A:cholesterol acyltransferase (ACAT). Addition of LDL to the culture medium stimulated this rate seven- to tenfold. Addition of TMD at 10 μg/ml stimulated the basal rate by 80% ($P = 0.002$). Higher doses of TMD tended to be inhibitory, with 30% inhibition at 20 μg/ml ($P < 0.05$) and 50% inhibition at 40 μg/ml ($P = 0.03$). However, LDL retained the ability to stimulate cholesterol esterification by seven- to tenfold at all concentrations of TMD. Thus, the inhibition of cholesterol esterification at 40 μg/ml TMD, both in the presence and the absence of LDL, appears to be due to a direct inhibition of ACAT and not due to an interruption of substrate supply. At a lower dose (20 μg/ml), which

TABLE 3. Effect of TMD on the secretion of bile acids by HepG2 cells

Treatment	Bile Acid Secretion (ng/10^7 cells/h)	
	Total Bile Acids	Mass Increase[a]
Control	57.3 ± 3.0[b]	
+ LDL	69.8 ± 2.8	12.5
+ TMD	7.5 ± 0.5	
+ TMD + LDL	18.5 ± 1.9	11.0

Confluent HepG2 cells in 100-mm culture dishes were incubated for 24 h in LPDS medium and then re-fed 8 ml of phenol red-free MEM containing either no addition (Control), LDL (100 μg/ml), TMD (40 μg/ml), or both LDL and TMD. Culture media were harvested after a 48-h incubation for the extraction and assay of bile acids as described under Experimental Procedures.

[a] Mass increase refers to the increase in bile acid secretion (ng/10^7 cells/h) observed due to the presence of LDL in the culture medium.

[b] The values are the average ± range of duplicate cultures for each treatment. The distribution of individual bile acids in control incubations was: chenodeoxycholic acid, 73%; cholic acid, 22%; 3α.7α, 12α-trihydroxy-5β-cholestan-26-oic acid, 4%; and 3α, 7α-dihydroxy-5β-cholestan-26-oic acid, <1% and was unaltered by any of the treatments.

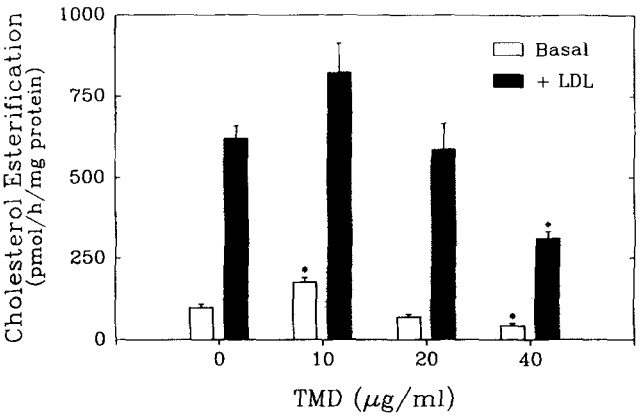

Fig. 4. Effect of TMD on cellular cholesterol esterification in HepG2 cells in the presence or the absence of LDL. Confluent HepG2 cells in 35-mm culture dishes were incubated with LPDS medium for 24 h and were then re-fed 2 ml of LPDS medium ± LDL (100 μg protein/ml) and the indicated amounts of TMD. After 20 h, 10 μCi of [^{3}H]oleate (200 nmol) complexed to bovine serum albumin was added to the culture medium and the cells were incubated for 2 h at 37°C. Radioactive cholesteryl oleate was extracted from the cells and measured as described under Experimental Procedures. The data represent the mean ± SEM of triplicate experiments (n = 9) at each concentration of TMD. *$P < 0.05$ versus the corresponding zero TMD control.

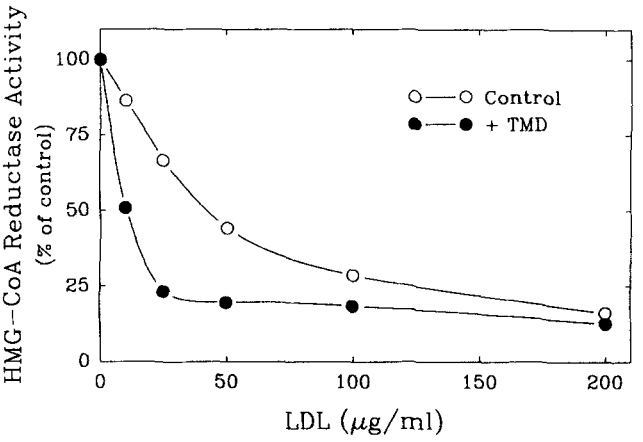

Fig. 5. Effect of TMD on the down-regulation of HMG-CoA reductase activity of HepG2 cells by LDL. Confluent HepG2 cells in 35-mm culture wells were incubated in LPDS medium for 24 h and were re-fed fresh LPDS medium ± TMD (40 μg/ml). After 2 h, indicated amounts of LDL were added to triplicate dishes in each group. After 24 h incubation, cells were harvested for the determination of HMG-CoA reductase activity (n = 3) as described under Experimental Procedures. The control (100%) values for enzyme activity (nmol mevalonate formed/h per mg protein) in the absence and the presence of TMD were 10.4 and 15.2, respectively.

is sufficient to abolish cholesterol biosynthesis, TMD had no deleterious effect on LDL-stimulated esterification of cholesterol.

A possible effect of TMD on the ability of LDL-derived cholesterol to enter a regulatory pool was investigated by examining the suppression of HMG-CoA reductase activity of HepG2 cells in response to varying amounts of LDL in the presence or the absence of TMD (**Fig. 5**). The results show that LDL is a more effective suppressor of reductase activity in HepG2 cells treated with TMD (40 μg/ml). The concentration of LDL required for 50% reduction of enzyme activity was lowered from 50 μg/ml to 10 μg/ml in TMD-treated cells.

The major route of export of cholesterol from HepG2 cells is secretion in the form of LDL- and HDL-sized particles (14, 15). We examined the effect of TMD on this process by measuring the rates of secretion of apolipoprotein B and of cholesterol (**Table 4**). No inhibition of apoB secretion could be observed in the presence of TMD. Treatment with TMD caused 30–40% enhancement of total cholesterol secretion in both control cells and those preloaded with LDL. Upon statistical analysis, this enhancement was found to be marginally significant ($P = 0.054$).

DISCUSSION

The data presented in this article demonstrate that TMD is a specific inhibitor of oxidosqualene cyclase in cultured HepG2 cells. TMD treatment caused an accumulation of squalene epoxides at the expense of newly synthesized cholesterol. The lack of inhibition of synthesis of total nonsaponifiable lipids from acetate indicates that TMD did not adversely affect any steps prior to squalene formation in the pathway. These findings are similar to the ones observed in studies with CHO fibroblasts (17, 18). The activity of HMG-CoA reductase, the rate-limiting enzyme of isoprenoid biosynthesis, showed only minor changes in TMD-treated cells. The 30% inhibition of reductase activity ($P < 0.05$) at 10 μg/ml TMD may be ascribed to the action of 24(*S*),25-epoxycholesterol which accumulates at this concentration of TMD (Fig. 1) and is a known suppressor of HMG-CoA reductase in cultured cells (34, 35). Similarly, the 30% stimulation ($P < 0.05$) at higher doses of TMD may be attributed to an inhibi-

TABLE 4. Effect of TMD on the secretion of apolipoprotein B and cholesterol by HepG2 cells

Preincubation	TMD	Apolipoprotein B	Total Cholesterol
		μg/mg cell protein/24 h	
LPDS	–	5.2 ± 0.8	3.17 ± 0.56
LPDS	+	5.5 ± 0.7	4.38 ± 0.69[a]
LDL	–	N.D.	5.11 ± 0.36
LDL	+	N.D.	6.67 ± 0.77[a]

Confluent HepG2 cells in 100-mm culture dishes were re-fed LPDS medium with or without 50 μg/ml LDL. After 24 h of preincubation, monolayers were rinsed three times with Saline G and were re-fed serum-free MEM ± TMD (40 μg/ml). Culture media from triplicate dishes for each treatment were harvested after 24 h for determination of apoB and cholesterol content as described under Experimental Procedures. The results are the average ± SEM (n = 6 and 12 for measurement of apoB and cholesterol secretion, respectively). N.D., not determined.

[a]Different from corresponding minus TMD control, $P = 0.054$.

tion of formation of endogenous sterol regulators (18). The absence of general toxic effects of TMD on HepG2 metabolism is further evidenced by the observation that the synthesis of neither fatty acids nor total proteins was affected.

Concentrations of TMD that abolished sterol biosynthesis also enhanced LDL uptake by two- to threefold. The extent of maximal induction appeared to be similar to that seen with the treatment of cells with lovastatin which inhibits a step common to the synthesis of both sterol and nonsterol products. This observation may be contrasted with the findings on the induction of HMG-CoA reductase in CHO cells by TMD (18). In CHO cells, total inhibition of sterol biosynthesis by TMD caused only a 50% enhancement of reductase activity whereas inhibition of all isoprenoid biosynthesis by 3-fluoromevalonate produced a much greater fourfold induction of enzyme activity, thus indicating the involvement of nonsterol regulators in reductase expression. The data in Table 1 show that a specific block in sterol formation is sufficient for full induction of LDL receptors in HepG2 cells and that a blanket inhibition of all isoprenoid biosynthesis is not necessary. The data also demonstrate that TMD has no adverse effects on the processing of internalized LDL. These results are consistent with the hypothesis that the regulation of LDL receptor expression in HepG2 cells may be carried out entirely by sterols.

Despite the increase in LDL receptor expression, the level of total cholesterol in cells incubated with both TMD and LDL compared to cells incubated with LDL alone is not significantly different ($P = 0.36$). This appears to be the result of a combination of a lack of biosynthesis and a normal or enhanced rate of secretion of cholesterol. The finding of normal rates of apoB secretion in TMD-treated cells suggests that excess cholesterol is repackaged into lipoproteins and effluxes from the cells. Forty-50% of cholesterol secreted from HepG2 cells is reported to be associated with apoB-containing particles (14, 15, 36). Craig and Cooper (36) have also reported that delivery of cholesterol to HepG2 cells by β-VLDL enhances the secretion of cholesterol and cholesterol esters in all classes of lipoproteins. Since we did not size-fractionate medium cholesterol into various lipoprotein classes, it is possible that TMD may have promoted the secretion of cholesterol in more than one class of lipoproteins. On the other hand, given the low rate of bile acid secretion by HepG2 cells, it is unlikely that bile acid-associated cholesterol contributed to a significant degree to the observed efflux either in the presence or the absence of TMD.

The reduction in the secretion of bile acids by TMD-treated HepG2 cells in the absence of an apparent inhibition of the bile acid biosynthetic pathway may be interpreted as due to a lack of available cholesterol in the substrate pool. Since newly synthesized cholesterol is thought to be the preferred substrate for bile acid biosynthesis (9, 30, 31), inhibition of cholesterol synthesis by TMD should be expected to cause a decrease in bile acid synthesis and secretion. Such an inhibition as a result of compactin or lovastatin treatment has been observed previously in both cultured rat hepatocytes (37) and in the intact rat (38–40). Acute inhibiiton of late steps of cholesterol biosynthesis in the livers of rats with compound AY-9944 was also shown to inhibit bile acid secretion (41). Supplementation of either untreated or TMD-treated HepG2 cells with LDL caused only a small increase in bile acid secretion. It is not clear whether TMD has any direct inhibitory effects on the bile acid biosynthetic pathway. As stated previously, the extremely low expressed activity of cholesterol 7α-hydroxylase in HepG2 microsomes (32) precluded an examination of a possible inhibitory effect of TMD on the activity of this rate-limiting enzyme of bile acid biosynthesis. However, in preliminary experiments, TMD, up to 40 μg/ml, did not inhibit cholesterol 7α-hydroxylase activity of rat liver microsomes in vitro (data not shown). The fact that the mass increase in bile acid secretion in HepG2 cells incubated with LDL is similar in the presence or the absence of TMD indicates that a relatively small portion of LDL-derived cholesterol enters the bile acid substrate pool in HepG2 cells and that the contribution of that cholesterol to bile acid synthesis is not affected by TMD. Given the low basal rate of secretion of bile acids by HepG2 cells, conversion to bile products may be quantitatively a minor route of metabolism for LDL-derived cholesterol in these cells.

We also investigated other pathways of cholesterol metabolism in TMD-treated HepG2 cells. While higher doses (>20 μg/ml) of TMD may be inhibitory to ACAT, they did not appear to prevent its stimulation by LDL, suggesting normal availability of LDL-derived cholesterol to the enzymes of endoplasmic reticulum. Inhibition of HepG2 cholesterol synthesis by compactin is also reported not to affect the ability of LDL to stimulate ACAT activity (38). In addition, Sandoz compound 58-035, an inhibitor of ACAT, did not affect bile acid synthesis in rat hepatocytes (38). These and other authors (42) have suggested that in liver cells the ACAT substrate pool of cholesterol may be different from the metabolically active pool of cholesterol which is the substrate for lipoprotein and bile acid synthesis. Our studies suggest that the secretion of lipoprotein cholesterol may actually be increased by TMD treatment of HepG2 cells. Additionally, treatment with TMD (40 μg/ml) potentiated down-regulation of HMG-CoA reductase activity of HepG2 cells by exogenous LDL. This finding is similar to the one obtained with Sandoz 58-035 (33), suggesting that by inhibiting ACAT, TMD allowed more of LDL-derived cholesterol to enter the regulatory pool. The lack of adverse interference with regulatory action of LDL distinguishes TMD from other late stage inhibitors of cholesterol biosynthesis (19).

The results of the current study clearly demonstrate that in HepG2 cells, TMD ($\geq$20 μg/ml) abolishes sterol biosynthesis by specifically inhibiting the activity of oxidosqualene cyclase. The activity of HMG-CoA reductase is either unchanged or even stimulated, suggesting that the synthesis of nonsterol derivatives of mevalonate is uninterrupted under these conditions. The fact that LDL uptake is induced by TMD to the same extent as lovastatin indicates the involvement of only sterol regulators in controlling LDL receptor expression. Further experiments have shown that in TMD-treated HepG2 cells utilization of the internalized cholesterol for bile acid synthesis, esterification, regulation, and efflux is comparable to that in untreated cells. These findings suggest that inhibitors specific for the sterol branch of the isoprenoid biosynthetic pathway such as TMD may have the potential to serve as anti-hypercholesterolemic agents.

We acknowledge the skillful technical assistance of Leonor Wenger, Shiela Miller, and Scott Blumenreich. This work was supported by a grant from the American Heart Association of Colorado (S.R.P.) and by grants DK42270 (S.R.P.) and HL23083 (T.A.S.) from the National Institutes of Health. This is contribution #1074 from the Eleanor Roosevelt Institute.

Manuscript received 16 April 1991 and in revised form 2 July 1991.

REFERENCES

1. Brown, M. S., P. T. Kovanen, and J. L. Goldstein. 1981. Regulation of plasma cholesterol by lipoprotein receptors. *Science.* **212:** 628–635.
2. Goldstein, J. L., and M. S. Brown. 1984. Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate plasma cholesterol. *J. Lipid Res.* **25:** 1450–1461.
3. Kovanen, P. T., D. W. Bilheimer, J. L. Goldstein, J. J. Jaramillo, and M. S. Brown. 1981. Regulatory role of hepatic low density lipoprotein receptors in vivo in the dog. *Proc. Natl. Acad. Sci. USA.* **78:** 1194–1198.
4. Ma, P. T., G. Gil, T. C. Sudof, D. W. Bilheimer, J. L. Goldstein, and M. S. Brown. 1986. Mevinolin, an inhibitor of cholesterol synthesis, induces mRNA for low density lipoprotein receptor in livers of hamsters and rabbits. *Proc. Natl. Acad. Sci. USA.* **83:** 8370–8374.
5. Panini, S. R., D. H. Rogers, and H. Rudney. 1985. Regulation of HMG-CoA reductase and the biosynthesis of nonsteroid prenyl derivatives. *In* Regulation of HMG-CoA Reductase. B. Preiss, editor. Academic Press, New York, NY. 149–181.
6. Gupta, A., R. C. Sexton, and H. Rudney. 1986. Modulation of regulatory oxysterol formation and low density lipoprotein suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by ketoconazole. *J. Biol. Chem.* **255:** 8348–8356.
7. Kempen, H. J., K. van Son, L. H. Cohen, M. Griffioen, H. Verboom, and L. Havekes. 1987. Effect of ketoconazole on cholesterol synthesis and on HMG-CoA reductase and LDL-receptor activities in Hep G2 cells. *Biochem. Pharmacol.* **36:** 1245–1249.
8. Gupta, A. K., R. C. Sexton, and H. Rudney. 1990. Differential regulation of low density lipoprotein suppression of HMG-CoA reductase activity in cultured cells by inhibitors of cholesterol biosynthesis. *J. Lipid Res.* **31:** 203–215.
9. Turley, S. D., and J. M. Dietschy. 1988. The metabolism and excretion of cholesterol by the liver. *In* The Liver: Biology and Pathology. I. M. Arias, W. B. Jacoby, H. Popper, D. Schacter, and D. A. Shafritz, editors. Raven Press, New York, NY. 617–641.
10. Havekes, L., V. Van Hinsbergh, H. J. Kempen, and J. Emeis. 1983. The metabolism in vitro of human low-density lipoprotein by the human hepatoma cell line HepG2. *Biochem. J.* **214:** 951–958.
11. Dashti, N., G. Wolfbauer, E. Koren, B. Knowles, and P. Alaupovic. 1984. Catabolism of human low density lipoproteins by human hepatoma cell line Hep G2. *Biochim. Biophys. Acta.* **794:** 373–384.
12. Wang, S. R., M. Pessa, J. Infante, D. Catala, C. Salvat, and R. Infante. 1988. Lipid and lipoprotein metabolism in Hep G2 cells. *Biochim. Biophys. Acta.* **961:** 351–363.
13. Everson, G. T., and M. A. Polokoff. 1986. A human hepatoblastoma cell line exhibiting defects in bile acid synthesis and conjugation. *J. Biol. Chem.* **261:** 2197–2201.
14. Thrift, R. N., T. M. Forte, B. E. Cahoon, and V. G. Shore. 1986. Characterization of lipoproteins produced by the human liver cell line, Hep G2, under defined conditions. *J. Lipid Res.* **27:** 236–250.
15. Ellsworth, J. L., S. K. Erickson, and A. D. Cooper. 1986. Very low and low density lipoprotein synthesis and secretion by the human hepatoma cell line Hep-G2: effects of free fatty acid. *J. Lipid Res.* **27:** 858–874.
16. Nelson, J. A., M. R. Czarny, T. A. Spencer, J. S. Limanek, K. R. McCrae, and T. Y. Chang. 1978. A novel inhibitor of steroid biosynthesis. *J. Am. Chem. Soc.* **100:** 4900–4902.
17. Chang, T. Y., E. S. Schiavoni, Jr., K. R. McCrae, J. A. Nelson, and T. A. Spencer. 1979. Inhibition of cholesterol biosynthesis in Chinese hamster ovary cells by 4,4,10β-trimethyl-*trans*-decal-3β-ol. A specific 2,3-oxidosqualene cyclase inhibitor. *J. Biol. Chem.* **254:** 11258–11263.
18. Panini, S. R., R. Schnitzer-Polokoff, T. A. Spencer, and M. Sinensky. 1989. Sterol-independent regulation of 3-hydroxy-3-methylglutaryl-CoA reductase by mevalonate in Chinese hamster ovary cells. *J. Biol. Chem.* **264:** 11044–11052.
19. Rudney, H., R. C. Sexton, A. Gupta, and S. R. Panini. 1987. Regulation of isoprenoid biosynthesis: oxygenated sterols as modulators of HMG-CoA reductase activity. *Chem. Scr.* **27:** 57–62.
20. Burki, E., J. Logel, and M. Sinensky. 1987. Endogenous sterol synthesis is not required for regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by low density lipoprotein. *J. Lipid Res.* **28:** 1199–1205.
21. Nelson, J. A., M. R. Czarny, and T. A. Spencer. 1982. Enzymatic 7β hydroxylation of 4,4,10β-trimethyl-*trans*-decal-3β-ol (TMD). *Bioorg. Chem.* **11:** 371–381.
22. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* **98:** 241–260.
23. Hara, A., and N. S. Radin. 1978. Lipid extraction of tissues with a low toxicity solvent. *Anal. Biochem.* **90:** 420–426.
24. Drevon, C. A., A. D. Attie, S. H. Pangburn, and D. Steinberg. 1981. Metabolism of homologous and heterologous lipoproteins by cultured rat and human skin fibroblasts. *J. Lipid Res.* **22:** 37–46.
25. Panini, S. R., R. J. Lutz, L. Wenger, J. Miyake, S. Leonard, A. Andalibi, A. J. Lusis, and M. Sinensky. 1990. Defective elongation of fatty acids in a recessive 25-

hydroxycholesterol resistant cell line. *J. Biol. Chem.* **265:** 14118–14126.
26. Nakagaki, M., and F. Nakayama. 1979. Class separation of bile lipids by thin-layer chromatography. *J. Chromatogr.* **177:** 343–348.
27. Carlson, S. E., and S. Goldfarb. 1977. A sensitive enzymatic method for the determination of free and esterified tissue cholesterol. *Clin. Chim. Acta.* **79:** 575–582.
28. Curry, M. D., A. Gustafson, P. Alaupovic, and W. J. McConathy. 1978. Electroimmunoassay, radioimmunoassay, and radial immunodiffusion assay evaluated for quantitation of human apolipoprotein B. *Clin. Chem.* **24:** 280–286.
29. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
30. Björkhem, I., and A. Lowenhaupt. 1979. Preferential utilization of newly synthesized cholesterol as a substrate for bile acid biosynthesis. An in vivo study using $^{18}O_2$-inhalation technique. *J. Biol. Chem.* **254:** 5252–5256.
31. Turley, S. D., and J. M. Dietschy. 1981. The contribution of newly synthesized cholesterol to biliary cholesterol in the rat. *J. Biol. Chem.* **256:** 2438–2446.
32. Erickson, S. K., and P. E. Fielding. 1986. Parameters of cholesterol metabolism in the human hepatoma cell line, HepG2. *J. Lipid Res.* **27:** 875–883.
33. Havekes, L. M., E. C. M. De Wit, and H. M. G. Princen. 1987. Cellular free cholesterol Hep G2 cells is only partially available for down-regulation of low-density lipoprotein receptor. *Biochem. J.* **247:** 739–746.
34. Saucier, S. E., A. A. Kandutsch, F. R. Taylor, T. A. Spencer, S. Phirwa and A. K. Gayen. 1985. Identification of regulatory oxysterols, 24(*S*),25-epoxycholesterol and 25-hydroxycholesterol, in cultured fibroblasts. *J. Biol. Chem.* **260:** 14571–14579.
35. Panini, S. R., A. Gupta, R. C. Sexton, E. J. Parish, and H. Rudney. 1987. Regulation of sterol biosynthesis and of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured cells by progesterone. *J. Biol. Chem.* **262:** 14435–14440.
36. Craig, W. Y., and A. D. Cooper. 1988. Effects of chylomicron remnants and β-VLDL on the class and composition of newly secreted lipoproteins by HepG2 cells. *J. Lipid Res.* **29:** 299–308.
37. Davis, R. A., P. M. Hyde, J-C. W. Kuan, M. Malone-McNeal, and J. Archambault-Schexnayder. 1983. Bile acid secretion by cultured rat hepatocytes. Regulation by cholesterol availability. *J. Biol. Chem.* **258:** 3661–3667.
38. Kempen, H. J. M., J. de Lange, M. P. M. Vos-van Holstein, P. van Wachem, R. Havenga, and R. J. Vonk. 1984. Effect of ML-236B (compactin) on biliary excretion of bile salts and lipids, and on bile flow in the rat. *Biochim. Biophys. Acta.* **794:** 435–443.
39. Björkhem, I. 1986. Effects of mevinolin in rat liver: evidence for a lack of coupling between synthesis of hydroxymethylglutaryl-CoA reductase and cholesterol 7α-hydroxylase activity. *Biochim. Biophys. Acta.* **877:** 43–49.
40. Pandak, W. M., D. M. Heuman, P. B. Hylemon, and Z. R. Vlacevic. 1990. Regulation of bile acid synthesis. IV. Interrelationship between cholesterol and bile acid biosynthesis pathways. *J. Lipid Res.* **31:** 79–90.
41. Pandak, W. M., Z. R. Vlacevic, D. M. Heuman, and P. B. Hylemon. 1990. Regulation of bile acid synthesis. V. Inhibition of conversion of 7-dehydrocholesterol to cholesterol is associated with down-regulation of cholesterol 7α-hydroxylase activity and inhibition of bile acid synthesis. *J. Lipid Res.* **31:** 2149–2158.
42. Suckling, K. E., and E. F. Stange. 1985. Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism. *J. Lipid Res.* **26:** 647–671.