Primary rat hepatocyte culture cells were used to study the acute regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in response to 25-hydroxycholesterol, 3β,5α,6β-cholestantriol, and mevalonolactone. All three effectors caused a rapid suppression of HMG-CoA reductase activity. 25-Hydroxycholesterol also caused an increase in the ratio of newly synthesized methyl sterols to newly synthesized C27-sterols. Furthermore, in 25-hydroxycholesterol-treated cells, the relative contribution of Δ24-sterol precursors to the nonsaponifiable lipid fraction increased. Di- and trimethyl-diene sterols were the dominant methyl sterols synthesized in the presence of 25-hydroxycholesterol.

3β,5α,6β-Cholestantriol (50 μM) also caused a very strong (97%) suppression of sterol demethylation; 4,4-dimethylmonoenone sterols were more prominent (23%) in cells treated with 3β,5α,6β-cholestantriol, than in cells treated with 25-hydroxycholesterol (2%). The rates of both unesterified and esterified sterol synthesis increased as a function of exogenous mevalonolactone concentration. C27-sterol synthesis was saturated at a concentration of (R)-mevalonolactone which produced only a 33% suppression of HMG-CoA reductase activity. However, there was a direct relationship between the accumulation of methyl sterols and the decrease in HMG-CoA reductase activity.

With the aid of triparanol, it was demonstrated that the suppression of HMG-CoA reductase activity by mevalonolactone was linked with the ability of the cells to convert squalene-2,3-epoxide into sterols. The results described in the present article support an important and perhaps necessary relationship between the rate of methyl sterol conversion of C27-sterols and the suppression or inhibition of HMG-CoA reductase in primary hepatocyte culture cells.

Mammalian cells require cholesterol for survival (1–3) and their rate of sterol synthesis can be modulated in response to extracellular cholesterol (4, 5). The extent of sterol synthesis modulation is dependent on cell type (6–12) and the quantity of cholesterol presented (4, 6, 12). This cholesterol must be metabolically active, that is, when sterol synthesis is blocked, exogenous cholesterol must be presented to the cell in a form which can support life (2, 10).

It has been established that a key regulated reaction in the synthesis of sterol from acetyl units is the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate (13, 14). This reaction is catalyzed by the enzyme HMG-CoA reductase (EC 1.1.1.34). Generally, modulation of HMG-CoA reductase activity is directly related to changes in the overall rate of cholesterol synthesis (13, 16).

Immunochromatographic studies from this laboratory (17) and those of Higgins and Rudney (18, 19) suggest that the regulation of HMG-CoA reductase may occur by an alteration of its catalytic activity, or a change in the number of its molecules, or both. The mode of regulation appears to be related to the effector or experimental perturbation used (17–19).

In the last few years, it has become evident that selected auto-oxidation products of cholesterol caused a more rapid rate and extent of suppression of HMG-CoA reductase activity (sterol synthesis) than highly purified cholesterol (20). In addition, mevalonate, the first committed precursor in the synthesis of isoprenoid compounds, caused a very strong suppression of rat liver HMG-CoA reductase activity in isolated cells or intact animals (21–23). These observations with mevalonolactone suggest that endogenously synthesized cholesterol, one of its precursors or products may also modulate HMG-CoA reductase's activity.

We report on short term studies to delineate the mechanism(s) of regulation for HMG-CoA reductase activity in primary hepatocyte culture (PHC) cells incubated in the presence of 25-hydroxycholesterol, 3β,5α,6β-cholestantriol, and (RS)-mevalonolactone. The kinetics of radioactive acetate or mevalonate incorporation into squalene, methyl sterols, and C27-sterols were compared with the extent of HMG-CoA reductase suppression.

It was found that changes in the ratio of newly synthesized methyl sterols to newly synthesized C27-sterols accompany suppression of HMG-CoA reductase activity in response to 25-hydroxycholesterol, (RS)-mevalonolactone, and 3β,5α,6β-cholestantriol. Alterations in the ratio of newly synthesized methyl sterols: C27-sterols was evident within 20 min. In addition, we demonstrated that sterols must be formed for the
strong suppression of HMG-CoA reductase activity by (RS)-
mevalonolactone. Therefore, acute suppression of HMG-CoA
reductase activity and decreased conversion of methyl sterol
to C37-sterols may represent an integrated regulatory process
which governs the flow of carbon to cholesterol.

MATERIALS AND METHODS

Chemicals—(R)-[3-14C]Mevalonolactone was purchased from New
England Nuclear. In several experiments, e.g., Fig. 5, this substrate
was diluted with unlabeled (RS)-mevalonolactone to yield a substrate
of an appropriate specific activity. In all cases, e.g., Fig. 5, the meva-
lonolactone concentration reported is that of (R)-mevalonolactone.
Sodium [3H]acetate, (RS)-[2-14C]Mevalonolactone, and (RS)-[5-3H]
mevalonic acid (DBED salt) were also purchased from New England
Nuclear. dt-[3-14C]HMG-CoA was synthesized by the method of Gol-
sdorf and Pfitz (24) and purified by DEAE-cellulose column
chromatography (25). (RS)-[1-14C]Squalene-2,3-epoxide was prepared
by a modification (tromethamin intermediate was not isolated
and purified before epoxidation) of the method described by Van Tame-
len and Curphey (26). 25-Hydroxycholesterol and 3b,5a,6b-cholestan-
triol were purchased from Steraloids. Triparanol was obtained from
A. Wasmann, Milan, Italy (27). N,N-Timethyl 3,7,11-trimethyl
dodecanamine was a gift from Dr. M. J. Thompson (United States
Department of Agriculture, Beltsville, Md.). Tissue culture supplies
were obtained from the cell culture division of the California
Institute of Technology, Pasadena, Calif. Other reagents were of highest available
quality and obtained from standard sources.

Primary Hepatocyte Cell Culture—Primary hepatocytes were
isolated from 3- to 5-day-old regenerative liver rat by the method of
Russo et al. (28) and cultured in Dulbecco’s Modified Eagle’s Medium
(Grand Island Biological Co., Grand Island, N. Y.) with the following additions: 10% lipoprotein poor serum (29), penicillin (100 units/ml),
streptomycin (100 units/ml), and ascorbic acid (200 pg/ml). Hepat-
ocytes were plated on either 100 mm or 150 mm collagen-coated
plastic tissue culture dishes (30) (Falcon) which contained 5 and 15 ml
of media, respectively. 5.0 X 105 to 10.0 X 105 cells were seeded per plate.
The dishes were incubated at 37°C with 10% CO2, 5% air atmosphere,
and the medium was changed 3 to 4 h after plating. If cells were used
for longer than 30 h, the medium was changed again at 24 h after
plating.

Isolation of Microsomes and Determination of HMG-CoA Reduct-
ase Activity—Plates (2 X 150 mm) were pooled to give 10 to 15 mg
of cellular protein. The cells were washed, twice with ice-cold Dul-
becco’s phosphate-buffered saline (0.9% NaCl solution) (Grand Island Biological Co., Grand Island, N. Y.), and then dislodged with a rubber
policeman into 8.0 ml of a buffer which contained: 0.3
m potassium PO4, 0.03 m sodium EDTA, and 0.01 m diethio-
reitol, pH 7.5. This cell suspension was sonicated (0-4°C) with a Bronwill
Biosonik (Bronwill Scientific, Rochester, N. Y.) (25% of maximum
power) for 30 s (5). After the addition of 5% of maximum output for 15 s. Microsomes were isolated from the sonicate as
described by Heller et al. (31) for rat liver homogenates. Microsomal
pellets were gently rinsed with homogenization buffer and assayed
(25) immediately or stored in liquid nitrogen. Frozen microsomes
were assayed within 1 week. All assays were done at two different
protein concentrations as a test for linearity. HMG-CoA reductase
activity is expressed as picomoles of mevalonate formed/min/mg of
protein. Protein was determined by the method of Lowry et al. (33)
on trichloroacetic acid precipitates (31) of the microsomal sus-
Squalene-2,3-oxide was synthesized by Z. S. Han, University of
New Mexico.

Thin Layer Chromatography of Radioactive Lipids—Non-saponi-
fiable and neutral lipid fractions were resolved by TLC (Silica Gel G; Merck
6773) with heptane:ethyl acetate (80:25 v/v) as the respective develop-
ment solvents. One centimeter bands were removed and counted in Aquasol
(New England Nuclear) (29). Standards were run in separate lanes
and detected by charring. Further discrimination of the radioactivity in
the “sterol ester” band (C37-sterol plus methyl sterol esters and
squalene) was determined by its removal, extraction with a solution of
chloroform:methanol:bromothymol blue (75:25:1 v/v), and a evaporation of the pooled
extracts under a stream of nitrogen. The lipid residue was saponified,
extracted, and rechromatographed (heptane:ethyl acetate (4:1 v/v)).

Analytical Column Chromatography—Non-saponifiable and neu-
tral lipid samples (in toluene) were chromatographed on a short column
(1.2 X 5 cm) of silicic acid (Unisol, 200 to 325 mesh) as previously described (34). Fifteen fractions of 8.0 ml each were collected
with toluene as the eluting solvent and aliquots of each fraction were
assayed for radioactivity by liquid scintillation spectrometry (34). Fractions 3 to 6 (methyl sterols) were combined, reduced in
volume under a stream of nitrogen, and filtered through a Swinnny
filtration apparatus containing a fluoropore membrane with an or-
ganic prefilter. The filtrates were allowed to dryness. The instant
and then dissolved in an appropriate volume of filtered toluene for
analytical column chromatography (35). The fractions containing 27-carbon sterols (7-15) were treated in similar fashion. Squalene and sterol esters
were collected in the first two fractions.

Individual reverse-phase high pressure liquid chromatography
analyses of the C37 and neutral sterol fractions from the silicic acid
chromatography was carried out on a Waters Prep LC column. The
eluting solvent was 12% aqueous acetonitrile and fractions (0.63 ml/
15 s) were collected. After the addition of Aquasol (10 ml), radioactiv-
ity was measured by scintillation spectrometry. Further details of
apparatus and methods have been described elsewhere (35).

The HPLC system separated sterol classes and the radioactive
peaks obtained were assigned to known reference compounds with
similar retention times. Additional sterols with the same degree of
unsaturation, number of methyl groups, and hydroxyl groups, plus
the reference standard, may be associated with the radioactive peak.

Therefore, the specific compounds presented in the tables are to be
viewed as examples of the sterol class which had retention times
identical with the radioactive peaks eluted by HPLC.

Presentation of Results—All of the data presented in this manu-
script are representative of two to four independent studies with
different hepatocyte preparations. In addition, the values given in
the respective tables and figures for the amount of radioactivity applied
to the silicic acid and high pressure liquid chromatography columns
of cholesterol is the total incorporation of a radioactive precursor per
mg of cellular protein per unit of time. Counting efficiencies were 40
to 60% of 1H and 80 to 90% for 1C.

RESULTS

Characterization of Primary Hepatocyte Culture Cells—Fig. 1 shows the time-dependent changes of HMG-CoA reductase
activity in PHC cells during 6 h of incubation. During the initial 4 to 6 h after plating, HMG-CoA reductase activity increased
2- to 2.5-fold and then decreased during the next 8 to 12 h to a relatively stable base-line.

Since we did not want to evaluate the regulation of HMG-
CoA reductase activity under conditions in which it was rapidly changing in the cells, our experiments were performed
with cells maintained for 20 to 36 h in culture (represented by the
hatched bar in Fig. 1).

Effect of Cholesterol Derivatives—In Fig. 2 we compare the
effectiveness of 25-hydroxycholesterol, cholesteryl succinate,
and 3b,5a,6b-cholestantriol to suppress HMG-CoA reductase
activity in PHC cells. Cholesteryl succinate did not suppress
HMG-CoA reductase activity. However, 25-hydroxycholes-
terol and 3b,5a,6b-cholestantriol caused a significant suppres-
sion (29). In each case, carrier lipid was added to aid in identifi-
cation of the relevant compounds by thin layer chromatography. All
solvent evaporation were done under a stream of nitrogen.
Sterol Synthesis in Hepatocytes

Fig. 1. HMG-CoA reductase activity in PHC cells. Cells were prepared from 4-day regenerated liver as described by Bissel et al. (28) and plated in 150 mm Petri dishes which contained 15 ml of Dulbecco's Modified Eagle Medium plus 10% (v/v) lipoprotein-poor bovine serum (29). Incubations were at 37°C in a humidified atmosphere of 95% air, 5% CO₂. Two plates were harvested at the times indicated and processed for measurement of HMG-CoA reductase activity as described under "Materials and Methods." Each sample was assayed at two protein concentrations and the results shown as the means for two to four different cell preparations.

Fig. 2. Suppression of HMG-CoA reductase activity in PHC cells by sterols. Cells were isolated as described in Fig. 1. Medium was changed 3 h after plating and the cells were used 18 h later. Steroids were added in different aliquots from ethanol solutions to give the final concentrations noted. The final ethanol concentration was the same for all cultures and did not exceed 0.5% (this concentration of ethanol had no measurable effect on lipid synthesis nor HMG-CoA reductase activity). Cells were incubated for 4 h, and processed for measurement of HMG-CoA reductase activity as described under "Materials and Methods."

Fig. 3 shows that 3β,5a,6β-cholestantriol effectively caused the accumulation of tritium label from [3H]acetate in methyl sterols and it also suppressed total incorporation of acetate into nonsaponifiable lipids by 69%.

Since 3β,5a,6β-cholestantriol appeared to act at both pre- and postmevalonate steps in the cholesterol biosynthetic pathway, we decided to determine whether 25-hydroxycholesterol had similar or additional effects on the distribution pattern of newly synthesized nonsaponifiable lipids.

In Fig. 4 we present a thin layer chromatogram of the radioactive distribution pattern for the nonsaponifiable lipid fraction from PHC cells incubated with 25-hydroxycholesterol (6.2 µM for 30 min) and pulsed an additional 20 min with (R)-[3-14C]mevalonolactone. As previously reported for HTC cells (37) and other growing cells (2, 3) the total incorporation of radioactive mevalonolactone into nonsaponifiable lipids was similar in the presence or absence of 25-hydroxycholesterol (see legend of Fig. 4). However, the relative distribution of the radioactive compounds found in the nonsaponifiable fraction was significantly different. 25-Hydroxycholesterol caused a decrease in the relative amount of radioactivity in C₂₇-sterols (e.g. cholesterol, desmosterol, etc.) with a concomitant increase in radioactive methyl sterols (e.g. lanosterol, 4,4-dimethyl- and 4-monomethyl sterols).

Identification of Nonsaponifiable Lipids—Analytical silicic acid column chromatography (34) and high pressure liquid chromatography (HPLC) (35) were used to partially characterize the unesterified sterols formed in the presence of 25-hydroxycholesterol and 3β,5a,6β-cholestantriol. The results from a silicic acid column chromatographic analysis (34) are summarized in Table I. We confirmed our TLC results (Figs. 3 and 4); that is 25-hydroxycholesterol and 3β,5a,6β-cholestantriol caused an increase in the fraction of nonsaponifiable lipid radioactivity found as methyl sterols, with a
PHC cells were maintained for 18 h in 150 mm² dishes as described for Fig. 2. The sterols were added from stock 95% ethanol solutions to give a final concentration of 12 μM and 60 μM for 25-hydroxycholesterol and 3β,5α,6β-cholestantriol, respectively. Control dishes were given an equivalent volume of 95% ethanol (20 μl). After 3 h incubation, 2.5 mm sodium [3H]acetate (1 mCi) or 7.7 μM (R)-[3-14C]mevalonolactone (2 μCi) were added; the cells were pulsed for 60 min. The total nonsaponifiable lipid fraction was isolated as described under "Materials and Methods" and analyzed by analytical silicic acid chromatography (34). Radioactivity (cpm) applied to the column: [3H]acetate; control (316,000), 25-hydroxycholesterol (53,000); [14C]mevalonolactone; control (901,000), 25-hydroxycholesterol (689,000), 3β,5α,6β-cholestantriol (912,000). Cellular protein per plate ranged from 4.7 to 6.0 mg.

### Table I

**Overall conversion of (R)-[3-14C]mevalonolactone and sodium [3H]acetate into sterols by primary hepatocyte culture cells**

<table>
<thead>
<tr>
<th>Sterol fractions</th>
<th>Control</th>
<th>25-Hydroxycholesterol</th>
<th>3β,5α,6β-Cholestantriol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl sterols</td>
<td>33</td>
<td>14</td>
<td>46</td>
</tr>
<tr>
<td>C27-Sterols</td>
<td>63</td>
<td>84</td>
<td>39</td>
</tr>
<tr>
<td>Unknown sterols</td>
<td>4</td>
<td>2</td>
<td>15</td>
</tr>
</tbody>
</table>

### Table II

**Conversion of (R)-[3-14C]mevalonolactone into unesterified sterols by isolated primary hepatocyte culture cells. HPLC analysis**

The unesterified sterol fraction from two pooled dishes (150 mm²) of PHC cells, treated identically with those described for Table 1, were analyzed by analytical HPLC as presented under "Materials and Methods." Radioactivity (cpm) applied to the column: methyl sterols; control (11,050), 25-hydroxycholesterol (11,350), 3β,5α,6β-cholestantriol (17,250); C27-sterols; control (6,800), 25-hydroxycholesterol (10,100), 3β,5α,6β-cholestantriol (11,300). Cellular protein per plate ranged from 5.1 to 5.6 mg.

### Table III

**Possible Inhibition of Sterol Synthesis**

Concomitant decrease in radioactive C27-sterols. These changes were evident with either radioactive mevalonolactone or acetate as precursors. When radioactive acetate was used as a precursor, the total radioactivity found in the nonsaponifiable lipid fraction was always reduced 60 to 80%, whereas with radioactive mevalonolactone, the total incorporation was not significantly different from the control flask.

The C27-sterol and methyl sterol fractions, obtained by silicic acid column chromatography, were further resolved by reverse-phase HPLC analysis. The results are presented in Table II. The compounds listed in Table II are representative of the sterol class with retention times identical with the eluted radioactive peaks. 25-Hydroxycholesterol caused the accumulation of radioactivity in Δ^4-Δ^7-(diene)-methyl sterols as opposed to side chain saturated derivatives. In addition, the C27-sterol fraction was enriched with Δ^7-sterols. These results demonstrate that 25-hydroxycholesterol decreases the conversion of methyl sterol precursors to C27-sterols and also inhibits the reduction of the sterol Δ^7-bond. 3β,5α,6β-cholestantriol caused a 97% block in the conversion of methyl sterols to C27-sterols in PHC cells. In contrast to previous results with a cell-free liver extract (36), 3β,5α,6β-cholestantriol caused, in PHC cells, the accumulation of both Δ^4- and Δ^7-methyl sterols. Since the level of radioactivity in C27-sterols was low, we couldn't evaluate the completeness of the apparent inhibition of sterol Δ^7-reductase by the concen-

### Table IV

**Utilization of Mevalonolactone**

In some of our initial studies, (RS)-[5-3H]mevalonolactone, at a concentration of 250 μM, was used to measure sterol synthesis. However, this concentration of mevalonolactone, in contrast to acetate (2.5 mM), caused a marked increase in the ratio of methyl sterols to C27-sterols (Table IV). Since (RS)-mevalonolactone had been shown to be a potent modulator of HMG-CoA reductase activity in rats (21-23) and primary hepatocyte suspensions (21), we decided to test whether its inhibitory capacity was also correlated with a change in the ratio of newly synthesized methyl sterols to C27-sterols.

In Fig. 5A, we compare the effect of mevalonolactone concentration on its initial rate of incorporation into certain specific nonsaponifiable lipids (squalene, methyl sterols, and C27-sterols) in PHC cells. The apparent initial rate of C27-sterol synthesis was maximized at a concentration of (R)-mevalonolactone which caused a 32% suppression of HMG-CoA reductase activity in 4 h. However, increasing concentra-
PHC cells were isolated and incubated as described for Fig. 2. 25-Hydroxycholesterol (12 μM) was added to a series of flasks (150 mm²) to which sodium [3H]acetate (5 mM; 1 mCi) was added at 0 time, 30 min, and 150 min. The cells were incubated an additional 30 min and the total nonsaponifiable lipid fraction resolved by TLC as described under “Materials and Methods.” Radioactivity in the total nonsaponifiable lipid fraction plus the C27-sterol and methyl sterol bands were measured. HMG-CoA reductase activity was assayed in microsomes from two pooled dishes (150 mm²) harvested at 30, 60, and 180 min after the addition of 25-hydroxycholesterol. The control flasks were given a volume of 95% ethanol equivalent to that used to add the 25-hydroxycholesterol (50 μl). Total cellular protein per plate ranged from 8.0 to 7.3 mg.

Table III: Conversion of [3H]acetate into nonsaponifiable lipids by PHC cells incubated with 25-hydroxycholesterol

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reductase activity</th>
<th>% H in total nonsaponifiable lipid fraction</th>
<th>Sterols</th>
<th>Ratio</th>
<th>% methyl sterols / % C27-sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/mg</td>
<td>cpm/mg protein</td>
<td>[H]</td>
<td>Methyl-</td>
<td>C27-</td>
</tr>
<tr>
<td>Control</td>
<td>0-30 min</td>
<td>286</td>
<td>21,297</td>
<td>21</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>30-60 min</td>
<td>304</td>
<td>19,765</td>
<td>28</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>150-180 min</td>
<td>249</td>
<td>19,837</td>
<td>27</td>
<td>47</td>
</tr>
<tr>
<td>25-Hydroxycholesterol</td>
<td>0-30 min</td>
<td>250</td>
<td>20,258</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>30-60 min</td>
<td>127</td>
<td>12,638</td>
<td>42</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>150-180 min</td>
<td>57</td>
<td>7,040</td>
<td>54</td>
<td>24</td>
</tr>
</tbody>
</table>

Table IV: Comparison of sodium [3H]acetate and (RS) [5-3H] mevalonolactone incorporation into nonsaponifiable lipids by PHC cells

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Acetate (2.5 mM)</th>
<th>Mevalonolactone (250 μM)</th>
<th>% total radioactivity applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squalene</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Methyl sterols</td>
<td>30</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>C27-sterols</td>
<td>57</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Unknown polar sterols</td>
<td>7</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

The large accumulation in radioactive cellular methyl sterols (detected by thin layer chromatography) with increasing concentrations of mevalonolactone (Fig. 5), was confirmed by silicic acid column chromatography and high pressure liquid chromatography. In Table V, the profile of unesterified radioactive sterols formed by PHC cells pulsed with 7 and 400 μM mevalonolactone are compared (the compounds listed are representative of the sterol class with retention times identical with the eluted radioactive peaks).

Mevalonolactone at a concentration of 7 μM was used to obtain the “control” nonsaponifiable sterol pattern (similar results were obtained with 2.5 μM (R)-mevalonolactone). Mevalonolactone at a concentration of 400 μM caused a significant accumulation (increased from 9 to 65%) of Δ5-7-methyl sterols. Newly synthesized C27-side chain saturated sterols (e.g. cholesterol) decreased from 75 to 14% of the total unesterified sterol fraction.

The accumulation of methyl sterols, as opposed to C27-sterols, with increased levels of (R)-mevalonolactone was of even greater significance, relative to the studies with 25-hydroxycholesterol and 3β,5α,6β,8β-cholestantriol (Table II, Figs. 3 and 4), because it was coupled with an increased carbon flow to nonsaponifiable lipids. In Table VI we compare the apparent rate of mevalonolactone incorporation into methyl sterols and C27-sterols at two different mevalonate concentrations. It was assumed that the endogenous mevalonate pool did not cause a significant dilution of the radioactive substrate.

A 52-fold increase in mevalonolactone concentration led to a 17-fold increase in its rate of incorporation into the total nonsaponifiable lipid fraction. However, there was a 110-fold increase in its rate of incorporation into methyl sterols and only a 7-fold increase in its rate of incorporation into C27-sterols. Therefore, increasing concentrations of (R)-mevalonolactone caused a strong shift toward methyl sterol synthesis coupled with a greater absolute increase in methyl sterol mass.

The effect of mevalonolactone concentration on sterol ester synthesis (sterol moiety) is also compared in Table VI. At both mevalonate concentrations tested (7.7 and 400 μM), 3% of the total newly synthesized sterols were esterified. However, the incorporation of mevalonate into methyl sterols esters (as a percentage of the total sterol ester fraction), increased from 6% at 7.7 μM (R)-mevalonate to 21% at 400 μM (R)-mevalonate.

Although the relative increase in the rate of incorporation of mevalonolactone into sterol esters was large (12- and 50-fold for C27-sterol ester and methyl sterol ester, respectively), the change in mass was much greater for the total unesterified sterols. Total unesterified sterol increased from 1.87 to 29.70 nmol of mevalonate incorporated/h/mg of protein, while total esterified sterol increased from 0.065 to 0.968 nmol of mevalonate incorporated/h/mg of protein.

The above results demonstrate that high levels of mevalonolactone increase the formation of unesterified and esterified sterols in PHC cells; however, the greatest effect was on the
Fig. 5. Effect of (R)-mevalonolactone concentration on nonsaponifiable lipid synthesis and HMG-CoA reductase activity. A, cells were plated in 100 mm<sup>2</sup> plus 150 mm<sup>2</sup> dishes and used 20 h later. The 100 mm<sup>2</sup> plates were used to measure the rate of incorporation of (R)-[3-<sup>14</sup>C]mevalonolactone into C<sub>27</sub>-sterols, methyl sterols, and squalene. HMG-CoA reductase activity was measured in pooled microsomes from two (150 mm<sup>2</sup>) dishes. Aliquots of a stock (RS)-mevalonolactone solution (2 M) added to each plate to give the concentrations of the (R) isomer noted. A constant amount of (R)-[3-<sup>14</sup>C]mevalonolactone (2 &mu;i) was also added to those dishes used to measure the rates of squalene, methyl sterol, and C<sub>27</sub>-sterol synthesis. Radioactivity incorporation of HMG-CoA reductase activity were measured after 1 h and 4 h, respectively. Uninhibited HMG-CoA reductase activity was 183 pmol/min/mg of protein. Samples were processed as described for Figs. 2 and 3. B, methyl sterol formation as a function of mevalonolactone concentration as expressed by the Hill equation. V<sub>max</sub> = 12.04 nmol of mevalonate incorporation/h/mg of protein. This value for V<sub>max</sub> was normalized to a value of 100, and all other values for methyl sterol formation (v) were compared to this norm. The slope of the line obtained in this plot is 2.01. C, HMG-CoA reductase inhibition as a function of mevalonolactone concentration as expressed by the Hill equation. I<sub>50</sub> = 87.3% inhibition of HMG-CoA reductase; I = the per cent inhibition of HMG-CoA reductase obtained at a given mevalonate concentration. The slope of the line obtained in this plot is 1.97.

Table V

Effect of mevalonolactone concentration on newly synthesized unesterified sterol profile of PHC cells

<table>
<thead>
<tr>
<th>Sterol fractions</th>
<th>(R)-[3-&lt;sup&gt;14&lt;/sup&gt;C]Mevalonolactone</th>
<th>% radioactivity in unesterified sterol fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl sterols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lanosterol + 4,4-dimethyl-Δ&lt;sub&gt;24&lt;/sub&gt;-cholestadienol</td>
<td>7.0</td>
<td>56.0</td>
</tr>
<tr>
<td>4-Monomethyl-Δ&lt;sub&gt;24&lt;/sub&gt;-cholestadienol</td>
<td>2.0</td>
<td>9.0</td>
</tr>
<tr>
<td>4,4-Dimethyl-Δ&lt;sub&gt;24&lt;/sub&gt;-cholestadienol</td>
<td>1.0</td>
<td>8.0</td>
</tr>
<tr>
<td>4-Monomethyl-Δ&lt;sub&gt;24&lt;/sub&gt;-cholesterol</td>
<td>2.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Unsaturated methyl sterols</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>C&lt;sub&gt;27&lt;/sub&gt;-sterols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ&lt;sub&gt;22&lt;/sub&gt;-Cholestadienol</td>
<td>2.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Δ&lt;sub&gt;24&lt;/sub&gt;-C&lt;sub&gt;27&lt;/sub&gt;-sterols</td>
<td>7.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Side chain saturated C&lt;sub&gt;27&lt;/sub&gt;-sterols</td>
<td>75.0</td>
<td>14.0</td>
</tr>
</tbody>
</table>

Table VI

Incorporation of (R)-[2-<sup>14</sup>C]mevalonolactone into sterols

<table>
<thead>
<tr>
<th>(R)-Mevalonolactone</th>
<th>Non-saponifiable lipid fraction</th>
<th>C&lt;sub&gt;27&lt;/sub&gt;-Sterols</th>
<th>Methyl sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>nmol mevalonolactone incorporated/h/mg protein</td>
<td>Free</td>
<td>Ester</td>
</tr>
<tr>
<td>7.7</td>
<td>1.98</td>
<td>1.710</td>
<td>0.061</td>
</tr>
<tr>
<td>400.0</td>
<td>33.40</td>
<td>12,700</td>
<td>0.767</td>
</tr>
</tbody>
</table>

mass of unesterified methyl sterols synthesized. We have also demonstrated methyl sterol accumulation in the serum, liver, and carcass of rats incubated with (RS)-[2-<sup>14</sup>C]mevalonolactone (400 mg).<sup>4</sup>

Inhibition of Sterol Synthesis—In an attempt to evaluate the interdigitation of post-squalene reactions with the regulation of HMG-CoA reductase activity, we used triparanol (39) and N,N-dimethyl-3,7,11-trimethyl dodecanamine (40),

<sup>4</sup>C. Havel and J. A. Watson, unpublished data.
pounds have been shown to be Δ^{4-sterol reductase inhibitors (39-41)), as experimental probes. Although both of these compounds have been shown to be Δ^{4-sterol reductase inhibitors, we found that at a concentration of 20 μM they caused a significant decrease in radioactive acetate incorporation into sterols.

Detailed studies (data not presented) showed that triparanol, over the concentration range of 0 to 40 μM, led to (a) an increase (1.5- to 2.0-fold) in □[H]acetate incorporation into nonnonsaponifiable lipids, (b) a gradual increase (1.5- to 2.0-fold) in HMG-CoA reductase activity, (c) decrease in sterol synthesis (90 to 10%), and (d) the accumulation of radioactivity in the nonnonsaponifiable fraction as squalene-2,3-epoxide (Fig. 6).

Evidence for the product being squalene-2,3-epoxide was based on its co-migration on thin layer chromatograms with authentic chemically synthesized squalene-2,3 epoxide and the identical extent of conversion to methyl sterols and C_{27} sterols when the biosynthetic □[H]squalene-2,3-epoxide was mixed with authentic chemically synthesized □[H]squalene-2,3-epoxide.

**Figure 6.** Effect of triparanol and N,N-dimethyl 3,7,11-trimethyl dodecanamine on nonnonsaponifiable lipid synthesis. Cells were isolated and treated as described for Fig. 5. Triparanol and N,N-dimethyl 3,7,11-trimethyl dodecanamine were added as ethanol solutions to give a final concentration of 30 μM, an equivalent volume (30 μl) of 95% ethanol was added to the control dishes. After 3 h incubation, □[R]-[3-□C]mevalonolactone (7.7 μM) was added and the cells were pulsed for an additional 60 min. The cells were harvested and processed to obtain the nonnonsaponifiable lipid fraction as described for Fig. 3 and under “Materials and Methods.” Radioactivity applied: control (□ — □), 65,100 cpm; triparanol (△ — △), 63,210 cpm; N,N-dimethyl 3,7,11-trimethyl dodecanamine (○ — ○), 66,230 cpm. Cellular protein varied from 4.2 to 4.6 mg/plate.

**Table VII**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Reductase activity in Experiments</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol/min/mg of protein</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>268</td>
<td>200</td>
</tr>
<tr>
<td>(RS) Mevalonolactone</td>
<td></td>
<td>52</td>
<td>40</td>
</tr>
<tr>
<td>Triparanol</td>
<td></td>
<td>605</td>
<td>300</td>
</tr>
<tr>
<td>(RS)-Mevalonolactone plus triparanol</td>
<td></td>
<td>428</td>
<td>210</td>
</tr>
</tbody>
</table>

Since 30 μM triparanol effectively muted (90%) the flow of carbon to sterols, we decided to test whether the cyclization of squalene-2,3-epoxide was required for the (R)-mevalonolactone mediated suppression of HMG-CoA reductase activity. A summary of these studies is presented in Table VII.

**Discussion**

In these studies we used a normal rat hepatocyte culture model to study selected aspects of the acute regulation of sterol synthesis by certain exogenous effectors. Our system was a modification of that described by Bissel et al. (28). We added lipoprotein poor bovine serum, plus insulin, to Dulbecco Modified Eagle's as opposed to Liebovitz-15 (42) medium and used collagen-coated plates (30). These conditions have been reported to enhance (a) plating efficiency, (b) overall appearance of the cells, and (c) the maintenance of a wider spectrum of differentiated functions (43-45).

With PHC cells we confirmed the initial, unexplained, enhancement of sterol synthesis during the first 6 h noted by Edwards (46), Cooper (47), and Goh and Heimberg (48). Other investigators (46, 50) have not reported similar observations with these isolated rat liver systems. This is probably due to differences in incubation medium, overall cellular integrity, or length of the experiments, or both.

A number of investigators have reported that biologically significant morphological and metabolic events of isolated hepatocytes begin to normalize 3 to 6 h after plating (28, 43-45). Thus, the initial 4 to 6 h of freshly isolated cells may not represent the optimal period to assess subtle regulatory aspects of hepatic metabolism. Gross elements of control might be detected, but in the absence of an apparent metabolic steady state, interpretations would have to be guarded. Therefore, we recommend that PHC cells be used between 18 and 30 h after plating (Fig. 1).

It was observed (Fig. 2) that cholesteryl succinate did not suppress HMG-CoA reductase activity in PHC cells. However, Gould (22) has shown that HMG-CoA reductase activity from freshly prepared hepatocyte suspensions was partially suppressed by cholesteryl succinate. In addition, Bell et al. (37) have reported that HMG-CoA reductase activity rat hepatoma tissue culture (HTC) cells was inhibited by cholesteryl succinate.

In studies with [□H]cholesterol [□C]succinate (10 μM) it was shown that the amount of ester or its hydrolyzed products associated with PHC cells was 20% of that found for an equivalent number of HTC cells. In addition, 40 to 50% of the [□H]cholesterol associated with PHC cells was the unhydrolyzed substrate. This was in contrast to our studies with HTC cells, which showed that 95 to 98% of the cell associated [□H]cholesterol was the succinate ester (37).

Our investigations with 3β,5α,Rβ-cholantriol in PHC cells confirmed previous studies with cell-free rat liver homogenates (36), i.e. 3β,5α,Rβ-cholantriol caused the accumulation of methyl sterols (Figs. 3 and 4; Tables I and II). 3β,5α,6β-
Cholestantriol also caused the suppression of HMG-CoA reductase (Fig. 2). Kandutsch and Chen (51) have recently reported that 3β,5α,6β-cholestantriol caused a significant suppression of HMG-CoA reductase activity in mouse fibroblast and fetal rat liver cells. In addition, we have found that 3β,5α,6β-cholestantriol caused the dual suppression of sterol demethylation and mevalonate formation in HTC cells. Based on our earlier studies with HTC cells (37), 3β,5α,6β-cholestantriol is as effective an inhibitor as cholest-4-en-3-one. These effects of 3β,5α,6β-cholestantriol are independent of the presence or absence of low density (1.006 ≤ d ≤ 1.063) lipoproteins in the medium.

Our studies with PFC cells suggest that the metabolic effects of 25-hydroxycholesterol on sterol synthesis are much broader than previously reported by this laboratory (37) and by others (20, 52, 53). In the present study, we demonstrated that 25-hydroxycholesterol (i) suppressed HMG-CoA reductase activity (Fig. 2), (ii) decreased sterol demethylation (Fig. 4, Tables I and II), and (iii) inhibited the reduction of Δ7-sterol by 5-cholestane-3β-ol (Table II). The latter effect has not been evaluated with cell-free extracts. However, the studies of Councell et al. (54) with mono- and diazacholesterol derivatives suggested that compounds with electronegative groups at carbon-25 of cholesterol would be strong inhibitors of sterol Δ7-reductase.

25-Hydroxycholesterol also stimulates the incorporation of [14C]oleate (bound to bovine serum albumin) into sterol esters of PFC cells. This is in marked contrast to our previous studies with HTC cells (37). Increased esterification is detectable within 15 min after the addition of 25-hydroxycholesterol (5 μM); the relative increase in sterol ester synthesis was greater for newly synthesized methyl sterols than for C7-sterols. Hence, there are at least four reactions intimately related to the synthesis and storage of sterols that are modulated in PFC cells by 25-hydroxycholesterol, i.e. (i) suppression of HMG-CoA reductase, (ii) inhibition of sterol demethylation, (iii) inhibition of sterol Δ7-reductase, and (iv) stimulation of sterol esterification. These four effects can all be detected within 30 min after the addition of 25-hydroxycholesterol. Our observations emphasize the complexity of the "25-hydroxycholesterol response."

Since the 25-hydroxycholesterol modulated reactions of sterol metabolism are all catalyzed by enzymes associated with the endoplasmic reticulum, its effect on other microsomal enzymes was evaluated. We found that 3- nitroanisole O-demethylase (28) and uridine diphosphate glucuronosyltransferase (EC 2.4.1.17) activities from PFC cells incubated with 25-hydroxycholesterol (7 μM for 4 h) were unaltered relative to control cells. Therefore, broad effect of 25-hydroxycholesterol on reactions related to the synthesis and storage of sterols does not appear to result from a generalized perturbation of the endoplasmic reticulum membrane. A directed effect on specific membrane domains enriched with enzymes of sterol synthesis and storage is a possible mode of action of 25-hydroxycholesterol.

25-Hydroxycholesterol causes a decrease in the number of low density lipoprotein receptors (52, 55). In addition, DNA synthesis (56, 57), Rb transport (58), plus bulk phase endocytosis (59) are inhibited in fetal mouse liver and in L-cells by 25-hydroxycholesterol. However, the earliest time used to evaluate these "25-hydroxycholesterol effects" was 6 h after its addition. This is much later than the time frame (15 to 30 min after the addition of 25-hydroxycholesterol) in which the modulation of HMG-CoA reductase activity, or sterol synthesis, is commenced. In addition, the 25-hydroxycholesterol-mediated decrease of DNA synthesis, bulk phase endocytosis, and mitogen-induced lymphocyte differentiation can be muted by cholesterol or mevalonate (56, 60). Therefore, these metabolic alterations are probably not responsible for the primary events which modulate HMG-CoA reductase activity, C7-sterol formation, or sterol ester synthesis.

With increasing concentrations of mevalonolactone we showed that the suppression of HMG-CoA reductase activity correlated most closely with changes in the rate of incorporation of mevalonolactone into unesterified methyl sterols, but not into C7-sterols (Fig. 5A). There was a strong mathematical correlation between methyl sterol synthesis and HMG-CoA reductase suppression when these data were analyzed by the Hill equation (Fig. 5B and C). In both instances (methyl sterol formation and HMG-CoA reductase inhibition) a slope of 2 was obtained, consistent with a second order cooperativity relative to mevalonolactone's concentration.

In addition, the concentrations of mevalonolactone required to produce 50% of maximal methyl sterol formation and 50% of the maximum inhibition of HMG-CoA reductase were similar, 125 μM and 85 μM, respectively. Furthermore, the strong suppression of HMG-CoA reductase activity by mevalonolactone was muted by triparanol (Table VII). These observations strongly support a regulatory role in cholesterol synthesis for reactions distal to squalene-2,3-oxide and prior to C7-sterols, i.e. sterol demethylation.

Modulations in these reactions would be reflected by changes in the mass, or relative distribution of methyl sterols in the nonsaponifiable lipid fraction, or both. HPLC analyses of the unesterified methyl fraction suggested that biological conversion of di- or trimethyl (Δ7α)-cholestanediol to cholesterol and other C7-sterols, or both, was altered by 25-hydroxycholesterol (Table II), 3β,5α,6β-cholestantriol (Table II), and mevalonolactone (Table V).

Brown and Goldstein (61) have reported that 4,4,14-trimethyl-Δ7α-cholastadienol (lanosterol) suppressed HMG-CoA reductase activity in human fibroblasts. Preliminary efforts by this laboratory to modulate HMG-CoA reductase activity in PHC cells by purified (62) lanosterol have been unsuccessful. Langdon and Bloch (63) were also unable to decrease [14C]acetate incorporation into cholesterol by rats fed lanosterol. However, squalene and various C7-sterols caused an 80 to 95% suppression of [14C]acetate incorporation into cholesterol. Studies to assess the transport and metabolism of radioactive lanosterol by PHC cells are in progress (63).

A possible role for sterol demethylation in acute and long term regulation of cholesterol synthesis, or HMG-CoA reductase activity, or both, may be inferred from studies of other investigators (22, 64-68). Recently, Schreopfer et al. (69, 70) have shown that C-14 and C-15 oxygenated C7-sterols are as good or better suppressors of HMG-CoA reductase activity than 25-hydroxycholesterol. We suggest that these compounds could also act as inhibitors of C-32 (C-14, α-methyl group) demethylation of lanosterol or related cholesterol precursors.

Spence and Gaylor (71) have prepared a 190-fold purified cytosolic protein preparation which, in vitro, modulated a fraction of the catalytic activity of methyl sterol oxidase and HMG-CoA reductase. Ritter and Dempsey (72) have also described a cytosolic protein (sterol carrier protein) preparation which activates the microsomal metabolism of sterol intermediates. Recently, we purified sterol carrier protein (SCP) 1400-fold from rat liver cytosol (73), and demonstrated that it was required to activate microsomal sterol demethylation. Sterol carrier protein (SCP) will not substitute for
studies are in progress to further delineate whether this is a SCPz (73). In addition, we have demonstrated that pure SCPZ concentration on its metabolism by liver, kidney, and blood cells. Saponifiable lipids 5- to 15-fold faster than HTC or Chinese pendis on a cell's capacity to transport and metabolize it. For results described in the present article support an important and perhaps necessary relationship between the rate of methyl sterol, cholesterol ester, methyl sterols, or possibly cholesterol binds 25-hydroxycholesterol.' These observations may suggest regulatory ligand. Spence and Gaylor (71) have proposed a act as a proximal negative effector of mevalonate synthesis, suggested regulatory role in cholesterol metabolism.

Sterol demethylation, and related metabolic effects of the sterol ligands and the resultant protein ligand complex could and cause a suppression of HMG-CoA reductase activity. Depending on the concentration of (R)-mevalonolactone used, one can alter the relative distribution of nonsaponifiable lipids and cause a suppression of HMG-CoA reductase activity. Therefore, in addition to a limited measurement of the total incorporation of radioactive mevalonolactone into total nonsaponifiable lipids has been used as a criterion to determine whether reactions distal to mevalonolactone into total nonsaponifiable lipids must be brought into these analyses. As a matter of refinement must be brought into these analyses.

CoA reductase, PHC cells convert mevalonolactone to non-

Using a number of different experimental techniques, the results described in the present article support an important and perhaps necessary relationship between the rate of mevalonolactone formation and the suppression or inhibition of HMG-CoA reductase in primary hepatocyte culture cells. Additional studies are in progress to further delineate whether this is a causal relationship or not.

REFERENCES


* A. A. Kandutsch and T. J. Scallen, unpublished data.
Sterol Synthesis in Hepatocytes

Chem. 250, 4025–4027
Regulation of cholesterol synthesis in primary rat hepatocyte culture cells.
Possible regulatory site at sterol demethylation.
C Havel, E Hansbury, T J Scallen and J A Watson


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