7-Ketocholesterol

ITS EFFECTS ON HEPATIC CHEROSTEROGENESIS AND ITS HEPATIC METABOLISM IN VIVO AND IN VITRO*

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The effects of 7-ketocholesterol on cholesterol biosynthesis and metabolism in the rat were investigated using the isolated, perfused liver and the whole animal. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was rapidly inhibited in both systems. In the whole animal, HMG-CoA reductase was inhibited by 60 to 70% after feeding 0.1% or 0.5% 7-ketocholesterol in the diet for 18 h. However, after 66 h of continuous feeding inhibition was no longer significant suggesting rapid development of tolerance to the sterol. In animals that showed significant inhibition of reductase activity, the levels of liver total cholesterol and microsomal cholesteryl esters had increased. In animals which had developed a tolerance for the sterol, these levels were normal, but microsomal cytochrome P-450 had increased suggesting induction of monooxygenase systems for metabolism of the sterol. 7-Ketocholesterol appeared to have toxic effects when fed at levels of 0.5% in the diet as indicated by decreases in liver and body weights after 66 h.

In both the intact rat and the perfused liver 7-keto[14C]cholesterol was rapidly metabolized to more polar derivatives and to cholesterol itself, primarily in the microsomal fraction. In the perfused liver the sterol and its metabolites were rapidly excreted in bile. The proportion of radioactivity in more polar metabolites increased with time, thus providing an explanation for the rapid development of tolerance. These results suggest 7-ketocholesterol is not promising as a hypocholesterolemic agent although it is a useful probe in the investigation of cholesterol metabolism.

Cholesterol biosynthesis in the liver is known to be suppressed rapidly and almost completely in most species of experimental animals by feeding cholesterol. Moreover, cholesterol added either as low density lipoprotein or as an ethanolic solution was shown to inhibit cholesterol biosynthesis in human skin fibroblasts (1, 2), human leukocytes (3), and rat hepatoma cells (4). Furthermore, perfusion of isolated rat livers with lecithin-dispersed pure cholesterol as well as ceramides and to cholesterol itself, primarily in the microsomal fraction. In the perfused liver the sterol and its derivatives were rapidly excreted in bile. The proportion of radioactivity in more polar metabolites increased with time, thus providing an explanation for the rapid development of tolerance. These results suggest 7-ketocholesterol is not promising as a hypocholesterolemic agent although it is a useful probe in the investigation of cholesterol metabolism.

*S The abbreviation used is: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.
tery acetate was a gift of Dr. N. Hays. All other chemicals were analytical grade.

Preparation and Purification of Free 7-Ketocholesterol-7-Ketocholesterol acetate was purified by recrystallization from an ethanol solution and recrystallization from aqueous potassium carbonate as described by Barnett et al. (14). The resulting 7-ketocholesterol was recrystallized from aqueous methanol to give a product with melting point of 165–166°C; literature value 165–167°C (14). It was further purified by thin layer chromatography on Silica Gel H in a benzene/diethyl ether (1:1) system to remove traces of cholesterol. Thin layer chromatography on Silica Gel HF using the same developing system gave a single band under ultraviolet light and a single band on visualization with iodine vapor. Analysis by gas-liquid chromatography indicated that only 7-ketocholesterol was present. There was no detectable contamination.

Preparation of 7a- and 7b-Hydroxycholesterols-7-Ketocholesterol was reduced with sodium borohydride in ethanol solution. After termination of the reaction (decrease of absorbance at 237 nm to essentially base-line) water was added and the 7-hydroxysterols extracted with petroleum ether. The a and b isomers were separated by thin layer chromatography on Silica Gel H, developing the plate three times in diethyl ether (15). The location of the sterols was visualized by iodine vapor. Authentic samples of 7-ketocholesterol and cholesterol were run on the same plate to locate these possible contaminants. The bands containing the a and b isomers were scraped from the plate and the sterols eluted with chloroform. Each sterol was checked for purity by thin layer chromatography in the benzene/diethyl ether (1:1) system and in diethyl ether (15), and by gas-liquid chromatography; the latter indicated more than 99% purity.

Radiochemical Purity of Labeled Sterols-Samples of labeled sterols were analyzed by thin layer chromatography on Silica Gel H using two different developing systems, benzene/diethyl ether (1:1) and diethyl ether (15). Authentic samples of cholesterol or 7-ketocholesterol were run on the same plate to locate these possible contaminants. The bands containing the a and b isomers were scraped from the plate into siliquix vials and counted in toluene/Liquifluor as described below. Positions of the authentic sterols were visualized under ultraviolet light and with iodine vapor, and the Rf values calculated. In all cases, the radiochemical purity of [3H]cholesterol used in experiments was >95%, and of 7-keto[3H]cholesterol, >99%.

Preparation of 7-Keto[3H]cholesterol—[3H]Cholesterol (labeled in 7b-hydroxysterol) was oxidized to 7-keto[3H]cholesterol acetate with chromium trioxide-d-butyl alcohol reagent (16). The ether was recrystallized from ethanol and hydrolyzed with potassium carbonate (16). The resulting 7-Keto[3H]cholesterol was recrystallized from aqueous methanol and further purified by thin layer chromatography on Silica Gel H 254, developing three times with benzene/diethyl ether (1:1). Authentic 7-ketocholesterol was run on the same plate. The plates were visualized under ultraviolet light and the band corresponding to 7-ketocholesterol scraped from the plate and the sterol eluted with chloroform. This material was checked for radiochemical purity by thin layer chromatography in the systems described above. The 7-keto[3H]cholesterol used in experiments was <0.05% radiochemically pure by thin layer chromatography.

Preparation of ur-[3-3H]CoA (supplied by Gofin and Pilo (17). It was converted to the acylglycine according to Goldfine and Pilo (17) and purified by chromatography in butanol/acetic acid/water as described by Low et al. (18). The specific activity of the ur-[3-3H]CoA was 2.087 Ci/mmol.

Animals-Male Sprague-Dawley rats weighing 250 to 300 g at time of killing were used in all experiments. Rats were housed individually under normal or reverse lighting (lights on 4 p.m.; off, 4 a.m.) and a 12-h light/dark cycle. The cholesterol biosynthesis peaked 6 h after the beginning of the dark phase. Rats were maintained on a commercial rat chow and water ad lib. The rats were allowed at least 10 days to stabilize the circadian rhythm of cholesterol biosynthesis before beginning an experiment.

Feeding Experiments-For each experiment, three pairs of rats were matched by weight, one of each pair was given a ground rat chow containing either 0.05%, 0.1%, or 0.5% 7-ketocholesterol, and the other was given grown rat chow alone. Both were allowed free access to water. The diets were prepared by adding the sterol in a large volume of perfusate-free diethyl ether to the ground rat chow, stirring, and allowing to dry in air at room temperature. For each animal, the amount of food consumed was measured, and for feeding experiments longer than 18 h the cholesterol level was also weighed as described by Barnett et al. (14).

Intragastric Administration of 7-Ketocholesterol—Doses of 6 to 10 mg of labeled or unlabeled 7-ketocholesterol were dissolved in diethyl ether, 1.0 ml of corn oil added, and the ether removed under nitrogen. The mixture was administered by stomach tube to lightly etherized rats. Control animals were given corn oil alone.

Preparation of Microsomes—The animals were killed close to the midpoint of the dark period by decapitation. The blood was collected for preparation of serum and the livers rapidly excised and placed in 0.9% saline NaCl solution at 4°C. All further steps were at 4°C unless otherwise indicated. Each liver was weighed, 0.5- to 1.0-g portions were separated for saponification, and the remainder minced and homogenized in 2 volumes (w/v) of Buffer I (0.1 M sucrose, 0.05 M KCl, 0.03 M EDTA, 0.04 M KH2PO4, pH 7.2) in a Potter-Elvehjem homogenizer by ten strokes at moderate speed. The homogenate was centrifuged twice for 10 min at 12,000 x g. The floating fatty layer was removed after each centrifugation, and the supernatant centrifuged for 60 min at 105,000 x g. The microsomal pellet from each liver was resuspended by homogenization in 8.0 ml of buffer by gentle homogenization with a loose-fitting Teflon pestle.

The assay was based on that of Shapiro et al. (20) as modified by Heller and Shrewsbury (21). Essentially, 1.0 to 2.0 mg of microsomal protein were added to 270 ml containing 10 mM dithiothreitol, 30 mM EDTA, 70 mM NaCl, 2 enzyme units of glucose-6-phosphate dehydrogenase, and 3 mM MgCl2 and the volume brought to 700 ml with water. The mixture was incubated with shaking for 20 min at 37°C. DL-3HMG-CoA (300 nmol) was added and the incubation continued for another 20 min. The reaction was stopped by the addition of 0.5 ml of 10 mM sodium hydride. The labeled mevalonolactone formed was separated from the reaction mixture and assayed according to Edwards and Gould (22) except that a saturating amount of sodium sulfate and RS-3Hmevalonolactone (92,000 dpm) as internal standard were added before diethyl ether extraction.

Determination of Microsomal Cytochrome P-450 Content—Microsomes were prepared as above from control livers. Aliquots were preincubated with a range of 7-ketocholesterol concentrations (10-4 to 10-6 M final concentration; added in ethanol) for 70 min at 37°C. Other aliquots were preincubated with the same concentration of ethanol and with no additions as controls. HMG-CoA reductase activity was assayed as above. In addition, aliquots of 10,000 g supernatant from control liver homogenates prepared as above were incubated for 1 h at 37°C with a range of 7-ketocholesterol concentrations as above. The appropriate controls as above were also incubated in the same way. Microsomes were isolated and washed as above and HMG-CoA reductase activity assayed as above. Total lipid extracts of those microsomes were also made to determine whether 7-ketocholesterol was present. The extracts were analyzed by thin layer chromatography as described below.

Preparation of Homogenates and Assay of Conversion of [3H]Acetate and [3H]Mevalonate to Digitonin-precipitable Sterols—The synthesis of [3H]-labeled digitonin-precipitable sterols from [3H]acetate or [3H]mevalonolactone was assayed essentially as described by Gould and Sweryd (24) using homogenates prepared in a medium based on that of Bucher et al. (25). For each group of liver, 3 volumes of oxygenated Bucher’s medium (0.1 M potassium phosphate, 4.0 mM MgCl2, 1.0 mM EDTA, 30 mM nicotinamide, pH 7.4) were added, the liver minced with scissors, and homogenized by 10 strokes at moderate speed. The homogenate was centrifuged for 45 min at 11,500 x g. The floating fatty layer was removed, and the supernatant centrifuged for 45 min at 11,500 x g. The floating fatty layer was removed, and the supernatant centrifuged for 45 min at 11,500 x g. The floating fatty layer was removed, and the supernatant centrifuged for 45 min at 11,500 x g. The floating fatty layer was removed, and the supernatant centrifuged for 45 min at 11,500 x g. The floating fatty layer was removed, and the supernatant centrifuged for 45 min at 11,500 x g. The floating fatty layer was removed, and the supernatant centrifuged for 45 min at 11,500 x g. The floating fatty layer was removed, and the supernatant centrifuged for 45 min at 11,500 x g.
was transferred to a glass centrifuge tube and the flask rinsed with 2 ml of water and 2 ml of 2 N perchloric acid. The contents were mixed and centrifuged for 10 min at 2,000 × g. The supernatant was decanted, 1 mg of carrier cholesterol added, and the pellet saponified with petroleum ether/diethyl ether/glacial acetic acid (75:25:1) in a glass-Teflon homogenizer. The 105,000 × g supernatant from the preparation of microsomes was centrifuged for 1 h at 105,000 × g and the floating, lipid-rich layer removed. The clear infranatant carefully separated from any sediment was denoted cytosol. Marker enzymes assayed were glucose-6-phosphatase (29) for endoplasmic reticulum, 5'-nucleotidase (30) for plasma membrane, and cytochrome oxidase assayed as hem a (31) for mitochondria. For determination of radioactivity in the fractions, aliquots were counted in Biofluor as described below.

Perfusion of Rat Livers in Vivo—The livers of rats weighing 160 to 200 g were perfused for periods of 0.5 to 3 h as described by Cooper (5).

After pentobarbital anesthesia, the liver was isolated in situ by the method of Mortimer (32). The portal blood flow was interrupted at most for 30 s during the operation of the organ. Oxygenation was accomplished with a silastic coil as described by Hamilton et al. (33) and tubing for the entire apparatus was discarded after each perfusion to prevent bacterial contamination.

The perfusate was composed of 22% washed rat red cells in Krebs-Ringer bicarbonate buffer containing 100 mg of glucose/100 ml. The perfusate was warmed to 37° and the pH adjusted to 7.4. The perfusate was circulated at the rate of 1.1 ml/min/g of liver. The total volume was 80 ml. Perfusate was equilibrated with 95% O₂ + 5% CO₂ when perfusion was established. Viability of the liver was judged by color, ability to extract O₂, and absence of gross perfusate loss.

Extraction and Separation of Metabolites of 7-Keto[3H]cholesterol—Aliquots of homogenates or subcellular fractions prepared as above were extracted with chloroform/methanol (2:1) (34). The total lipid extract was fractionated on Silica Gel HF 254 by thin layer chromatography. Two different developing systems were used for each sample: benzene/diethyl ether (1:1) and diethyl ether (15). Authentic samples of cholesterol, cholesteryl palmitate, 7-ketocholesterol, 7-ketocholesterol acetate, 7-cholestanol, and 7α-hydroxycholesterol were also plated. Each plate was developed three times, visualized under ultraviolet light, the sample channels divided into 0.5 cm bands, scraped into scintillation vials, and counted in toluene/Liquifluor as described below. The plate was also visualized with iodoine vapor and the Rf values of all standards recorded.

Bile was extracted with chloroform/methanol (2:1) according to Folch et al. (35). The total lipid extract (chloroform phase) was analyzed as described above and the methanol-water phase was fractionated by thin layer chromatography on Silica Gel Q using system I of Hofmann (36) with sodium taurocholate, sodium taurochenodeoxycholate, and sodium glycocholate as standards.

Identification of [3H]Cholesterol as Metabolite of 7-Keto[3H]cholesterol—Livers which had been perfused with 7-keto[3H]cholesterol were homogenized and aliquots were treated in two ways. (a) One aliquot was saponified with alcoholic KOH and the nonsoniponifiable material extracted with petroleum ether, and the residue fractionated by thin layer chromatography on Silica Gel II using petroleum ether/diethyl ether/glacial acetic acid (75:25:1). After visualization with iodoine vapor, the bands with the same Rf as an authentic sample of cholesterol on the same plate were scraped off the plate and eluted with chloroform. The residual crude cholesterol was taken up in 1.0 ml of ethanol and designated as Sample A. (b) A second aliquot of homogenate was extracted with chloroform/methanol and petroleum ether. The total lipid extract was fractionated on Silica Gel H using benzene/diethyl ether (1:1). The free cholesterol band was scraped off the plate, eluted with chloroform, and the residual crude cholesterol taken up in 1.0 ml of ethanol. This was designated Sample B.

Small aliquots of Samples A and B, each containing about 500 μg of cholesterol, were triturated in triplicate by thin layer chromatography in each of the three following systems: petroleum ether/diethyl ether/glacial acetic acid (75:25:1), benzene/diethyl ether (1:1), and diethyl ether. Each plate was developed three times and the cholesterol band was then scraped off the plate and assayed for tritium.

Additional aliquots of Samples A and B containing about 500 μg of cholesterol were fractionated by two dimensional thin layer chromatography according to Smith et al. (37). Authentic cholesterol samples were run on a separate plate but under the same conditions.

All other hands made visible by iodoine vapor were also scraped off the plates and counted but none of them was found to have a significant amount of tritium.

[3H]Cholesterol in bile was identified as follows. The residue of the chloroform phase of a total lipid extract of bile was fractionated by thin layer chromatography on Silica Gel H with benzene/diethyl ether (1:1). After visualization with iodoine vapor, the band with the same Rf as cholesterol was scraped from the plate and eluted with chloroform. It was taken to dryness under nitrogen and the residue dissolved in acetone. Aliquots were taken for determination of radioactive activity, cholesterol, and for analysis by thin layer chromatography as described above.

Chemical Methods—To determine total cholesterol, aliquots of whole blood, liver, spleen, and microsomes were saponified with alcoholic KOH and extracted with petroleum ether (38). To determine free and esterified cholesterol, aliquots of microsomes were extracted with chloroform/methanol (2:1) (34) and the total lipid extracts fractionated by thin layer chromatography on Silica Gel H with petroleum ether/diethyl ether/glacial acetic acid (75:25:1). Bands corresponding to authentic cholesterol and cholesteryl esters were scraped from the plate and the sterol and its ester eluted with chloroform. Recovery of cholesterol and cholesteryl esters was 80 to 85%. Cholesterol was determined by the ferric chloride method of Leffler (39).

Protein was estimated by the biuret method (40) against bovine serum albumin as standard. In some cases protein was estimated according to Lowry et al. (41) using the same standard.

Estimation of Radioactivity—Samples were counted in toluene/Liquifluor for nonaqueous samples, in Bray’s scintillation fluid (42) for reductase assays, and in Biofluor for tissue samples using a Packard Tri-Carb scintillation counter. [3H]Cholesterol and [3H]carnitine standards were in the appropriate scintillation fluid to estimate counting efficiency.

Gas Liquid Chromatography—Gas liquid chromatography was performed with a Varian model 2400 gas-liquid chromatograph with a 6-foot column packed with 5% OV17 on 90/100 mesh Gas-Chrom Q. Gas chromatographic conditions were: column temperature was 290°. The retention times were: 7α-cholestanol (reference standard), 6.0 min; cholesterol, 16.9 min; 7α-hydroxycholesterol, 38.6 min; 7-ketocholesterol, 62.7 min.

RESULTS

Effect of 7-Ketocolesterol on HMG-CoA reductase in Isolated Perfused Liver—The isolated perfused liver system previously described by Cooper (5) was used to study 7-ketocolesterol effects on HMG-CoA reductase activity in the intact liver. Perfusions with a serum-free medium resulted in a consistent increase in HMG-CoA reductase activity after 3 h (Table I).

The addition of 7-ketocolesterol (6 mg/100 ml of perfusate) to the perfusate gave final reductase activities that were significantly lower than those of the control perfusions (p < 0.001). Addition of smaller amounts of 7-ketocolesterol resulted in variable degrees of suppression. Addition of pure cholesterol to the perfusate as an equimolar dispersion with lecithin at a final concentration of 20 mg/100 ml also inhibited reductase activity as least as potently as 7-ketocolesterol (Table I), but comparison of the dose-response to 7-ketocolesterol with that to cholesterol is difficult because of differing stabilities of the two sterols in lecithin dispersions. When cholesterol was added to the perfusate in ethanol at 6 mg/100 ml no suppression of reductase activity was observed. However, from the work of Jakoi and Quarfordt (43) addition of 20 mg of cholesterol dispersed 1:1 with lecithin should
provide about 6 mg available for transfer to the liver, which is the same as the dose of 7-ketocholesterol found to be inhibitory.

Effect of 7-Ketocholesterol on Hepatic HMG CoA Reductase — In order to assess the effect on reductase activity of 7-ketocholesterol in vivo, groups of rats were fed a control diet or one to which 7-ketocholesterol had been added. Animals were killed after 18 h or 66 h and their hepatic HMG-CoA reductase activities determined. When 0.5% 7-ketocholesterol was fed for 18 h, a mean decrease of 73% in hepatic reductase activity was found (Table II). A 0.1% 7-ketocholesterol diet gave similar results (63% inhibition), but a diet containing 0.05% resulted in only 30% inhibition which was not statistically significant. For comparison, 0.1% cholesterol in the diet for 18 h resulted in 49% inhibition of reductase and 7.5% cholesterol, in 80% inhibition (Table II).

When animals were fed 0.1% 7-ketocholesterol in the diet for 66 h, however, HMG-CoA reductase activity was no longer significantly inhibited (Table II). Only a few animals were tested with the 0.5% regimen for 66 h because of the apparent toxic effects (see below); this group still showed some inhibition (46%) but it was not statistically significant.

To determine whether or not 7-ketocholesterol inhibits hepatic cholesterol biosynthesis primarily by suppression of HMG-CoA reductase activity, incorporation of [1'4C]acetate into digitonin-precipitable sterols was compared with that of [1'4C]mevalonate in liver homogenates from control and 7-ketocholesterol-fed rats. Animals fed a 0.1% 7-ketocholesterol diet for 18 h showed a 69% decrease in acetate incorporation and a 60% decrease in HMG-CoA reductase activity (Table III). Mevalonate incorporation was reduced by only 18% confirming that HMG-CoA reductase is the primary site for the inhibitory effect of 7-ketocholesterol on hepatic cholesterogenesis in rats as was found for cells in tissue culture (7–9).

**Table I**

<table>
<thead>
<tr>
<th>Dietary sterol concentration</th>
<th>No. of rats</th>
<th>HMG-CoA reductase activity</th>
<th>Inhibition and significance</th>
<th>Microsomal cholesterol</th>
<th>Increase in esterified %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmol/min/mg protein ± S.E.</td>
<td></td>
<td>Free/µg/mg protein ± S.E.</td>
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<tr>
<td>18 h of sterol feeding</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05% 7-ketocholesterol</td>
<td>6</td>
<td>1.40 ± 0.11</td>
<td>30; NS*</td>
<td>30 ± 2</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>2.14 ± 0.32</td>
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<td>28 ± 3</td>
<td>1.5 ± 0.2</td>
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<tr>
<td>0.1% 7-ketocholesterol</td>
<td>6</td>
<td>0.91 ± 0.08</td>
<td>63; p &lt; 0.002</td>
<td>25 ± 1</td>
<td>1.9 ± 0.1</td>
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<tr>
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<td>2.47 ± 0.28</td>
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<td>25 ± 1</td>
<td>1.2 ± 0.2</td>
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<td>0.5% 7-ketocholesterol</td>
<td>6</td>
<td>0.68 ± 0.13</td>
<td>73; p &lt; 0.001</td>
<td>24 ± 2</td>
<td>2.8 ± 0.5</td>
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<tr>
<td>Control</td>
<td>6</td>
<td>2.50 ± 0.20</td>
<td></td>
<td>26 ± 0.5</td>
<td>1.0 ± 0.1</td>
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<tr>
<td>0.1% cholesterol</td>
<td>3</td>
<td>0.98 ± 0.04</td>
<td>49; p &lt; 0.05</td>
<td>20.5 ± 1.2</td>
<td>2.0 ± 0.1</td>
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<tr>
<td>Control</td>
<td>3</td>
<td>2.00 ± 0.36</td>
<td></td>
<td>18.5 ± 0.5</td>
<td>0.9 ± 0.2</td>
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<td>7.5% cholesterol</td>
<td>2</td>
<td>0.37 ± 0.05</td>
<td>50; p &lt; 0.02</td>
<td>29 ± 4</td>
<td>5.0 ± 0.8</td>
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<td>Control</td>
<td>3</td>
<td>1.84 ± 0.05</td>
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<td>29 ± 4</td>
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<td>66 h of sterol feeding</td>
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<td>0.05% 7-ketocholesterol</td>
<td>3</td>
<td>1.89 ± 0.15</td>
<td>19; NS</td>
<td>26 ± 2</td>
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<tr>
<td>Control</td>
<td>2</td>
<td>2.34 ± 0.42</td>
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<td>0.1% 7-ketocholesterol</td>
<td>11</td>
<td>1.63 ± 0.16</td>
<td>10; NS</td>
<td>30 ± 1</td>
<td>2.2 ± 0.2</td>
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<tr>
<td>Control</td>
<td>11</td>
<td>1.81 ± 0.13</td>
<td></td>
<td>29 ± 1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>0.5% 7-ketocholesterol</td>
<td>3</td>
<td>0.88 ± 0.05</td>
<td>46; p &lt; 0.20</td>
<td>23 ± 1</td>
<td>2.5 ± 0.1</td>
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<tr>
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<td>3</td>
<td>1.04 ± 0.20</td>
<td></td>
<td>32 ± 1</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>0.1% cholesterol</td>
<td>3</td>
<td>1.37 ± 0.43</td>
<td>32; p &lt; 0.35</td>
<td>19.2 ± 0.4</td>
<td>1.7 ± 0.2</td>
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<tr>
<td>Control</td>
<td>3</td>
<td>2.00 ± 0.36</td>
<td></td>
<td>18.5 ± 0.5</td>
<td>0.9 ± 0.2</td>
</tr>
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</table>

* NS, not significant.
Three matched pairs of animals were used. One of each pair was fed 0.1% 7-ketocholesterol in the diet for 18 h before killing. Each liver was assayed individually. Homogenates were prepared from one-half of each liver as described under "Materials and Methods" and the incorporation of [14C]acetate or [14C]mevalonate into digitonin-precipitable sterols assayed as described under "Materials and Methods." Microsomes were prepared from the other half of each liver and HMG-CoA reductase activity assayed as described under "Materials and Methods." The values are given ± range.

<table>
<thead>
<tr>
<th>Group</th>
<th>Synthesis of digitonin-precipitable sterols from [14C]-labeled acetate</th>
<th>HMG-CoA reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/g liver/h</td>
<td>% inhibition</td>
</tr>
<tr>
<td>Control</td>
<td>3.66 ± 0.31</td>
<td>69</td>
</tr>
<tr>
<td>7-Ketocholesterol</td>
<td>1.12 ± 0.01</td>
<td>69</td>
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</table>

from these rats showed an abnormal lipoprotein pattern characterized by a second α-band on agarose gel electrophoresis.

**Effects on Serum, Liver, and Microsomal Cholesterol**

There was no discernible effect on serum cholesterol levels in any of the experimental groups with the exception of animals fed 0.5% 7-ketocholesterol for 66 h where a decrease of about 40% was found. After 18 h of feeding 7-ketocholesterol, total liver cholesterol had increased in those animals which showed inhibition of HMG-CoA reductase. Animals showing no inhibition of reductase activity did not have elevated total liver cholesterol levels.

The microsomal free cholesterol concentration showed no apparent change in animals fed 0.1% or 0.5% 7-ketocholesterol for 18 h, but the cholesteryl ester concentration increased (Table II). Data for control rats, those fed 7-ketocholesterol which did not show significant inhibition and those fed 7.5% cholesterol fell on a line (correlation coefficient -0.60, p < 0.01, Fig. 1). The steepness of the slope indicates a relationship between the reductase activity and the concentration of esterified cholesterol. Data for 7-ketocholesterol-fed rats which did show inhibition fell on a second, almost horizontal line (Fig. 1) suggesting that 7-ketocholesterol inhibition is probably not mediated by changes in microsomal esterified cholesterol.

**Effect of 66 H of Feeding of 0.1% 7-Ketocholesterol on Liver Microsomal Cytochrome P-450 Content**

The recovery of 7-Ketocholesterol from the liver for metabolizing or excreting 7-ketocholesterol. Therefore, the microsomal cytochrome P-450 content was measured as one index of the monoxygenating capacity of the liver. The cytochrome P-450 levels in experimental animals fed 0.1% 7-ketocholesterol for 66 h had increased to 1.20 ± 0.04 nmol/mg of protein compared with 0.80 ± 0.07 for controls (p < 0.001). There was no measurable change in liver weight.

**Hepatic Metabolism and Identification of Metabolites of 7-Ketocholesterol**

The increase in microsomal cytochrome P-450 levels observed during the disappearance of the inhibitory effect of 7-ketocholesterol on reductase activity suggested that the sterol might be metabolized to more polar compounds. The metabolism of the sterol was therefore investigated by the use of 7-keto[3H]cholesterol. Initially, 7-keto[3H]cholesterol was injected intraperitoneally into rats or given intragastrically 18 h before the rats were killed. However, very little radioactivity was recovered in livers from these animals, so the perfused liver system was used to study metabolism of the sterol and its excretion in the bile.

After perfusion of livers with 7-keto[3H]cholesterol for 2 h, the concentration in the liver of 7-ketocholesterol and its metabolites was 80 to 120 µg/g of liver. Of the total radioactivity recovered in the liver, 90% was recovered in the lipids. The components of this fraction were separated and identified by thin layer chromatography in two different systems (Table IV). About 70% of the recovered label migrated with the same RF as 7-ketocholesterol; the major detectable metabolite, constituting about 20% of the total, had the same RF as 7β-hydroxycholesterol. About 3% of the radioactivity was recovered in a band with the same RF, as cholesterol and was further fractionated by thin layer chromatography in four different solvent systems designed to separate mixtures of sterols (Table V). The 7-keto[3H]cholesterol preparation contained traces of [3H]cholesterol as a contaminant which must be considered as a possible source of the [3H]cholesterol isolated from the liver. Thin layer chromatographic analysis showed the contamination to be <0.08% of the 7-ketocholesterol. If it is assumed that all of the [3H]cholesterol perfused through the liver is taken up, it would account for less than 10% of the [3H]cholesterol isolated from the liver. These exper-

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2 The method of cholesterol determination used (39) gives no color reaction with 7-ketocholesterol so that observed increases in cholesterol levels actually reflect increases in cholesterol itself. Furthermore, separation of free and esterified cholesterol by thin layer chromatography separates both from the 7-keto- and 7-hydroxycholesterols and their esters.
7-Ketocholesterol Effects on Hepatic Cholesterogenesis

TABLE IV
Distribution of 7-keto\(^{3}H\)cholesterol and its metabolites in total lipid extracts of liver homogenates after perfusion with 7-keto\(^{3}H\)cholesterol

<table>
<thead>
<tr>
<th>Band corresponding to authentic samples of 7-ketocholesterol</th>
<th>Distribution of radioactivity in total lipid extract (chloroform phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl palmitate</td>
<td>Benzenediethyl ether (1:1)</td>
</tr>
<tr>
<td>7-Ketocholesterol acetate</td>
<td>1.3</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>3.1</td>
</tr>
<tr>
<td>7-Ketocholesterol</td>
<td>70.6</td>
</tr>
<tr>
<td>7β-Hydroxycholesterol</td>
<td>18.6</td>
</tr>
<tr>
<td>7α-Hydroxycholesterol</td>
<td>11.1</td>
</tr>
<tr>
<td>Unidentified</td>
<td>1.6</td>
</tr>
</tbody>
</table>

α 7α- and 7β-hydroxycholesterols were not clearly separated in this system.

TABLE V
Identification of radioactivity appearing in whole liver homogenates as cholesterol

Rat livers were perfused with 7-keto\(^{3}H\)cholesterol for 2 h as described under "Materials and Methods." Homogenates were prepared in Buffer 1 as described under "Materials and Methods." The cholesterol in each aliquot was separated by thin layer chromatography in the benzene-diethyl ether system or the petroleum ether/diethyl ether/glacial acetic acid system as described under "Materials and Methods." The band corresponding to authentic cholesterol was scraped from the plates and eluted with chloroform. The samples were dried under N\(_2\) and taken up in absolute ethanol. Aliquots were counted in toluene/Liquifluor and other aliquots were analyzed by thin layer chromatography as described under "Materials and Methods." Still other aliquots were assayed for cholesterol as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity in thin layer system*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>215</td>
</tr>
<tr>
<td>2</td>
<td>220</td>
</tr>
<tr>
<td>3</td>
<td>277</td>
</tr>
<tr>
<td>4</td>
<td>221</td>
</tr>
</tbody>
</table>

* System 1, benzene/diethyl ether, 1:1; 2, diethyl ether; 3, petroleum ether/diethyl ether/glacial acetic acid, 75:25:1; 4, ethyl acetate/acetone/heptane, 1:1, followed by acetone/heptane, 1:1, in second dimension.

TABLE VI
Comparison of distribution of 7-keto\(^{3}H\)cholesterol and its major metabolites in subcellular fractions of rat liver after perfusion with 7-keto\(^{3}H\)cholesterol for 30 min or for 2 h

The livers were perfused as described under "Materials and Methods" with the addition of 6 mg of 7-keto\(^{3}H\)cholesterol (specific activity: 0.6 mCi/mmol) to 100 ml of perfusate. Liver subcellular fractions were prepared and assayed as described under "Materials and Methods." Determination of marker enzymes (5'-nucleotidase, plasma membrane; glucose-6-phosphatase, endoplasmic reticulum; heme α, mitochondrial) indicated minimal (<10%) cross-contamination among the fractions. The conversion of 7-keto\(^{3}H\)cholesterol to more polar metabolites was determined by thin layer chromatographic analyses of total lipid extracts as described under "Materials and Methods." Two separate livers were represented in the data for 30 min, and four in the data for 2 h.

<table>
<thead>
<tr>
<th>Liver fraction</th>
<th>Specific radioactivity</th>
<th>7-keto(^{3}H)cholesterol converted to more polar compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>2 h</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>1597</td>
<td>3260</td>
</tr>
<tr>
<td>Microsomes</td>
<td>291</td>
<td>800</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>51</td>
<td>200</td>
</tr>
<tr>
<td>Cytosol</td>
<td>10</td>
<td>120</td>
</tr>
</tbody>
</table>

dpml/mg protein

after 1 h increasing to 40 ± 5% after 2 h (average of four separate animals).

Thin layer chromatography of the lipid material in bile indicated that much of the radioactivity in this fraction was recovered in a band with \(R_f\) corresponding to 7-ketocholesterol; in addition, a significant amount migrated with the some \(R_f\) as cholesterol (Table VI). Gas-liquid chromatography of material eluted from these bands gave major peaks (≥98% of total) with retention times corresponding to 7-ketocholesterol and cholesterol, respectively. The identity of the material migrating as cholesterol was confirmed further as described under "Materials and Methods."

Because of the small amount of material obtained, a defini-
tive analysis of the polar material in bile has not yet been possible. Preliminary analyses of these compounds by thin layer chromatography showed that about 50% of the labeled compounds in this phase migrated with Rf values similar to those of the major bile salts of the rat (Table VII).

**DISCUSSION**

In agreement with reports for a variety of mammalian cells in culture (6-11), 7-ketocholesterol produces a significant inhibition of hepatic HMG-CoA reductase activity, both in the perfused liver and in the intact rat, but there are important differences. First, the degree of suppression with 7-ketocholesterol was not nearly as complete as in these other systems; the greatest inhibition produced in vivo by nontoxic levels of the drug was 63% whereas in tissue culture cells it has been reported to be almost complete. Second, rats rapidly developed tolerance to 7-ketocholesterol in the diet. In contrast to the results in rats, Kandutsch et al. (44) have very recently reported that mice fed 7-ketocholesterol do not show significant inhibition of in vivo cholesterol synthesis in liver. Intestinal sterol synthesis was inhibited early but tolerance developed on prolonged feeding.

It has been known for many years that feeding cholesterol causes an increase in liver total cholesterol, including both free and esterified forms, and an inhibition of cholesterol biosynthesis (45-47). Since many of the enzymes of cholesterol biosynthesis and metabolism are localized in the microsomal fraction, changes in this cholesterol pool may be significant in modulating the rate of cholesterogenesis. Harry et al. (48) and Edwards and Gould (49) found that cholesterol feeding increased the level of esterified but not free cholesterol in microsomes suggesting a relationship between this increase and the concomitant decreased cholesterol biosynthetic rate. In the studies reported here, 7-ketocholesterol increased microsomal esterified cholesterol content in animals showing a decreased reductase activity. The reduction in HMG-CoA reductase caused by 7-ketocholesterol was greater than would be predicted by the degree of increase in microsomal cholesterol ester (Fig. 1) indicating that a different mechanism is probably involved.

A possible explanation of the increase in microsomal cholesterol ester observed on feeding 7-ketocholesterol is suggested by the report of van Cantfort (50) that 7-ketocholesterol is a potent inhibitor of microsomal cholesterol-7α-hydroxylase, the first and major rate-limiting step in bile acid synthesis. Thus, a failure of cholesterol catabolism induced by 7-ketocholesterol could result in rapid accumulation of microsomal cholesterol. Such an initially localized increase in cholesterol and/or cholesterol esters may rapidly modulate HMG-CoA reductase activity.

The rapid development of tolerance for 7-ketocholesterol observed in vivo was associated with an increase in liver microsomal cytochrome P-450 suggesting that monoxygenase systems might be involved. Cytochrome P-450 has been implicated in a number of steps in bile acid synthesis (51) and in many of the drug detoxification systems of the liver. If monoxygenase activities are involved in the development of tolerance one might expect 7-ketocholesterol to be converted to more polar derivatives.

In the perfused liver as well as in short term in vivo experiments, 7-keto[3H]cholesterol and its metabolites were detected throughout the cell. Distribution of the label suggested that the sterol was taken up via the plasma membrane followed by transport to the microsomes where it was metabolized. The major detectable product was 7β-hydroxycholesterol, in agreement with the observation of Mitton et al. (52) and Björkhem et al. (53) who found that the 20,000 × g supernatant of a rat liver homogenate or isolated microsomes converted 7-ketocholesterol to 7β-hydroxycholesterol and unidentified more polar products.

Analysis of the metabolites of 7-keto[3H]cholesterol always revealed radioactivity migrating with the same Rf as cholesterol. Although it is difficult to prove definitively that the radioactivity was cholesterol and not an unidentified compound with properties similar to cholesterol it could not be separated by the techniques used, the accumulated evidence strongly indicates that a few per cent of 7-ketocholesterol is actually converted to cholesterol itself. 7β-Hydroxy cholesterol is readily dehydrated to 7-dehydrocholesterol by heating and by chemical methods, and one may speculate that enzymes capable of catalyzing this reaction are present in liver. It is of interest that the cholesterol formed from 7-ketocholesterol seems to become part of a pool rapidly excreted into the bile as shown by its specific activity which was higher than that of total liver cholesterol.

We observed that 7-ketocholesterol and its metabolites were rapidly excreted in the bile during perfusion with 7-keto[3H]cholesterol, and that the proportion of polar metabolites in the bile increased with time. The major metabolite identified was 7β-hydroxycholesterol. Some of the polar metabolites may be bile salts but it cannot be assumed that they are normal ones; Björkhem et al. (53) concluded from studies with bile fistula rats fed 7-ketocholesterol that it was converted to abnormal bile acids. To date these polar metabolites of 7-ketocholesterol have not been identified definitively.

On the basis of this investigation, two important conclusions seem warranted. First, the inhibition of cholesterol biosynthesis by cholesterol feeding is not due to the presence of small amounts of the common oxygenated drivative 7-ketocholesterol, since the intact animal rapidly develops a tolerance for 7-ketocholesterol but apparently does not do so for cholesterol itself. Secondly, it is unlikely that this sterol will prove

### Table VII

**Distribution of radioactivity in bile after perfusion of rat livers with 7-keto[3H]cholesterol**

Livers were perfused for 1 h as described under "Materials and Methods" with the addition of 6 mg of 7-keto[3H]cholesterol (specific activity: 0.4 or 0.6 mCi/mmol) to 100 ml of perfusate. Bile was collected and extracted as described under "Materials and Methods." The chloroform and methanol phases of the total lipid extracts were analysed by thin layer chromatography as described under "Materials and Methods." Authentic samples of sterols or of bile salts were chromatographed on the same plates. After 1 h of perfusion, 76 ± 6% of the total radioactivity present in the bile was recovered in the chloroform phase decreasing to 60 ± 5% after 2 h.

<table>
<thead>
<tr>
<th>Band corresponding to authentic samples of</th>
<th>Distribution of radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Lipid phase</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>22.6</td>
</tr>
<tr>
<td>7-Ketocholesterol</td>
<td>18.8</td>
</tr>
<tr>
<td>7α- + 7β-hydroxycholesterols</td>
<td>4.9</td>
</tr>
<tr>
<td>Unidentified</td>
<td>27.0</td>
</tr>
<tr>
<td>Origin</td>
<td>26.7</td>
</tr>
<tr>
<td>B. Polar phase</td>
<td></td>
</tr>
<tr>
<td>Taurocholate</td>
<td>15</td>
</tr>
<tr>
<td>Taurochenodeoxycholate</td>
<td>21</td>
</tr>
<tr>
<td>Glycocholate</td>
<td>12</td>
</tr>
<tr>
<td>Unidentified</td>
<td>52</td>
</tr>
</tbody>
</table>

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useful in clinical applications as was suggested by others (11, 12) because of this rapid development of tolerance for the sterol at low dosages and the occurrence of toxic effects at higher levels (0.5% in the diet).

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