Studies on the link between HMG-CoA reductase and cholesterol 7α-hydroxylase in rat liver

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Abstract Under most experimental conditions, there is a covariation between the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase, and the rate-limiting enzyme in bile acid biosynthesis, cholesterol 7α-hydroxylase. The most simple explanation for the coupling between the two enzymes is that newly synthesized cholesterol is a substrate for an unsaturated cholesterol 7α-hydroxylase and that substrate availability is of major regulatory importance for this enzyme. The following results seem, however, to rule out that such a simple regulatory mechanism is of major importance and that HMG-CoA reductase activity per se is of importance in the regulation of cholesterol 7α-hydroxylase. *1*) The apparent degree of saturation of cholesterol 7α-hydroxylase, as measured in vitro in rat liver microsomes, was found to be relatively high (70–90%) under most experimental conditions, including starvation, cholestyramine treatment, and cholesterol treatment. A significant decrease in the degree of saturation was obtained first after a drastic reduction of total concentration of cholesterol in the microsomes by treatment with high doses of triparanol, an inhibitor of cholesterol biosynthesis. *2*) The stimulatory effect of cholesterol feeding on cholesterol 7α-hydroxylase activity in rats seems to be an effect on the enzyme activity (enzyme induction?) rather than an effect on substrate availability. Thus, the stimulatory effect of cholesterol feeding was retained also after almost complete removal of the endogenous cholesterol by extraction with acetone. *3*) Biliary drainage leads to a several-fold increase in the activity of both HMG-CoA reductase and cholesterol 7α-hydroxylase. The latter increase, however, cannot be due to the increased HMG-CoA reductase activity per se since infusion of cholesterol-enriched Intralipid to bile fistula rats led to a depressed HMG-CoA reductase activity with little or no effect on cholesterol 7α-hydroxylase. Similarly, depression of HMG-CoA reductase by use of mevalonate in the drinking water had little or no effect on cholesterol 7α-hydroxylase. It is concluded that microsomal cholesterol concentration, degree of substrate saturation, and levels of HMG-CoA reductase are not major direct regulators for cholesterol 7α-hydroxylase activity in rat liver.—**Björkhem, I., and J-E. Åkerlund.** Studies on the link between HMG-CoA reductase and cholesterol 7α-hydroxylase in rat liver. *J. Lipid Res.* 1988. **29:** 136–143.

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Under most experimental conditions, there is a covariation between the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase, and the rate-limiting enzyme in bile acid biosynthesis, cholesterol 7α-hydroxylase (for a review, see ref. 1). The reason for this very important covariation is poorly understood. Both enzymes may be regulated by the same factor(s), or the changes in the activity of cholesterol 7α-hydroxylase may be secondary to the changes in the activity of HMG-CoA reductase.

In one particular experimental situation, however, the activities of the two enzymes change in opposite directions. Thus, treatment of rats with dietary cholesterol leads to a depression of HMG-CoA reductase activity and a stimulation of cholesterol 7α-hydroxylase activity. The simplest explanation for the latter finding is that the cholesterol load leads to increased availability of substrate for cholesterol 7α-hydroxylase. If so, the coupling between HMG-CoA reductase activity and cholesterol 7α-hydroxylase activity observed under most conditions may be due to the fact that increased HMG-CoA reductase activity leads to increased supply of substrate for cholesterol 7α-hydroxylase.

There are some reports supporting this simple model for regulation of cholesterol 7α-hydroxylase. Mitropoulos et al. (2) reported that administration of mevalonic acid to rats led to an increased rate of 7α-hydroxylation of cholesterol. It was suggested that this increase could have been due to an increased intracellular pool of cholesterol in the environment of the HMG-CoA reductase that may have acted as a substrate for cholesterol 7α-hydroxylase (2). Shefer et al. (3) reported that cholesterol-free acetone powder prepared from rat liver microsomes obtained from rats fed a diet containing 2% cholesterol had about the same cholesterol 7α-hydroxylase activity as corresponding preparations from control rats (3).

Results from some other experiments are, however, difficult to explain. In a recent work from this laboratory, it was shown that treatment of rats with cholestanol, a substrate analogue to cholesterol, also led to an increased 7α-hydroxylation of cholesterol (4). This finding is better explained by an effect of the feeding on the enzyme activity than by an effect on the substrate availability. Results

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; DTT, dithiothreitol.

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of experiments in which the relative rates of 7α-hydroxylation of exogenous and endogenous cholesterol were measured by isotope dilution–mass spectrometry are also difficult to fit with a regulatory model where the substrate pool is of major importance (4, 5). In addition, there seems to be little or no correlation between the concentration of free cholesterol in the microsomes and cholesterol 7α-hydroxylase activity under several different conditions (5, 6). A prerequisite for a regulatory model, in which the substrate pool of cholesterol is of major importance, is that the cholesterol 7α-hydroxylase is unsaturated under the conditions employed. If the enzyme is fully saturated with substrate, changes in the substrate pool should be of little or no regulatory importance. In a study by Boström (7), a purified cytochrome P-450 LM_4 fraction with a high specific cholesterol 7α-hydroxylase activity was found to have a K_M towards cholesterol of 36 μM. Since the concentration of free cholesterol in the microsomes is in the millimolar range, it is difficult to believe that an enzyme with such a low K_M can be unsaturated to a degree allowing a threefold increase in activity as a consequence of an increased pool of substrate. On the other hand, the purified cholesterol-free enzyme may have properties different from those of the membrane-bound enzyme. In addition, it is always difficult to determine the degree of substrate saturation when the substrate is lipophilic and insoluble in aqueous medium.

In the present study, attempts were made to measure the degree of saturation of cholesterol 7α-hydroxylase in crude liver microsomes from untreated rats and rats subjected to different treatments. In addition, we have repeated the experiments by Shefer et al. (3), and assayed cholesterol 7α-hydroxylase activity in acetone powder prepared from untreated and cholesterol-treated rats. A highly accurate technique has been used for the assay, based on isotope dilution–mass spectrometry. We have also investigated the possibility that HMG-CoA reductase activity per se is of importance for cholesterol 7α-hydroxylase activity by selective depression of HMG-CoA reductase activity in bile-fistula rats and cholestyramine-treated rats in which both HMG-CoA reductase activity and cholesterol 7α-hydroxylase activity are stimulated. The results do not favor the hypothesis that substrate availability or HMG-CoA reductase activity are of major direct regulatory importance for cholesterol 7α-hydroxylase activity.

MATERIALS AND METHODS

Materials

[4-^{14}C]Cholesterol and [3-^{14}C]HMG-CoA with specific radioactivities of 55–60 mCi/mmol were obtained from The Radiochemical Centre (Amersham, Great Britain). The labeled cholesterol was purified by aluminum oxide chromatography immediately before use (8). 7α-[$^2H_3$]Hydroxycholesterol was prepared as described previously (8). Intralipid 10% was obtained from Kabi Vitrum (Stockholm, Sweden). To this Intralipid, additional 10% soybean oil was added, containing 50 mg of cholesterol per ml. The final concentration of cholesterol in the Intralipid was about 5.3 mg/ml. The mixture was mixed with an Ultraturax and sonicated to produce a stable emulsion. It should be pointed out that Intralipid is an emulsion with particles less than 1 μm. The cholesterol added to the Intralipid should dissolve in these particles. Triparanol was a kind gift from Merrell Dow Inc., Cincinnati, OH. All cofactors were obtained from Sigma Chemical (St. Louis, MO). All solvents used were of highest analytic grade.

Animals and animal treatments

Male rats (200–250 g) of Sprague-Dawley strain were used. With some exceptions (see Results) they were given free access to a commercial fat-free pellet diet. In some experiments (Table 1) the rats were starved for 15 hr prior to being killed. In some other experiments the rats were fed a diet containing 5% cholestyramine (Questran®, Bristol-Myers) for 6 days prior to being killed. In some cases the rats were fed a diet with 2% cholesterol for 3 days. The cholesterol was added to the diet dissolved in peanut oil, the latter being 10% of the diet. The control animals were given only the corresponding peanut oil diet without cholesterol. In some experiments the rats were given drinking water containing 12 mg of mevalonate per ml (corresponding to about 300 mg/24 hr) for 3 days. In some experiments the bile duct was cannulated under ether anesthesia. The animals were then kept in restraining cages for 48 hr prior to being killed. The corresponding control animals were sham-operated and then treated in the same way. The rats with a biliary fistula were given the ordinary pellet diet, but were given 0.6% (w/v) NaCl instead of water. In some experiments a central venous catheter was placed in the vena cava via the vena jugularis under ether anesthesia. These animals were infused with Intralipid enriched with cholesterol (see above) or with saline (0.9%, w/v) for 48 hr. In some experiments bile-fistula animals were also infused with cholesterol-enriched Intralipid or saline for 48 hr. In some experiments mevalonate, 500 mg/kg body weight, dissolved in 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, was injected intravenously into untreated rats or rats treated with cholestyramine. These rats were killed 1 hr after the injection.

The animal operations were approved by the Local Ethical Committee for animal experiments.

Preparations of subcellular fractions

Homogenates of rat liver were prepared in 50 mM Tris-Cl buffer, pH 7.4, containing 0.3 M sucrose, 50 mM NaCl, 10 mM EDTA, and 10 mM DTT (10% homogenate, w/v). A microsomal fraction was prepared by centrifugation at

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20,000 *g* for 15 min and recentrifugation of the supernatant at 100,000 *g* for 1 hr. Half of the microsomal fraction was resuspended in the homogenizing medium and recentrifuged at 100,000 *g* for 1 hr. This fraction was used for assay of HMG-CoA reductase. Half of the original microsomal fraction was recentrifuged at 100,000 *g* in a homogenizing medium lacking DTT. The resulting fraction was used for assay of cholesterol 7α-hydroxylase activity.

Preparations of smooth and rough microsomes

Microsomes were prepared from the liver of a starved rat under the conditions described by Bergstrand and Dallner (9) (20% homogenate in 0.25 M sucrose). The microsomes were then centrifuged in a sucrose gradient (1.3–0.6 M) containing 15 mM CsCl. The smooth fraction was recovered in the 0.6/1.3 gradient and the rough fraction at the bottom. The smooth fraction was diluted with water to a final sucrose concentration of about 0.25 M and recentrifuged for 1 hr at 105,000 *g*. The pellets corresponding to the smooth and rough fraction, respectively, were suspended in 0.15 M Tris-Cl, pH 8.0, and recentrifuged at 105,000 *g* for 1 hr. The resulting pellets were resuspended in the same medium as the microsomal fraction to give a total concentration of about 2 mg of protein/ml.

Preparation of cholesterol-free microsomal preparations (acetone powder)

Acetone powder of the microsomal preparations was prepared as described by Shefer et al. (3). The acetone powder thus obtained contained less than 1 μg of cholesterol/mg of microsomal protein, as compared to about 20 μg/mg in the original preparations. The catalytic activity of the acetone powder was similar to that reported by Shefer et al. (3), but was only 20–30% of that of the original microsomal preparation when assayed under identical conditions. Cholesterol 7α-hydroxylase activity was found to be linear with incubation time up to 30 min and with the amount of microsomal protein up to at least 40 mg. After addition of 150 μg of cholesterol, the enzyme was saturated with substrate.

Assay of cholesterol 7α-hydroxylase activity

After preparation of a microsomal fraction as above, incubations with 10 μg of [4-^{14}C]cholesterol dissolved in 1 mg of Tween 80 were performed as described previously in a total volume of 3 ml of 0.1 M potassium phosphate buffer, pH 7.4 (8). In some experiments the labeled cholesterol was replaced with various amounts of unlabeled cholesterol (0–300 μg). 7α-[$^{2}H_3$]Hydroxycholesterol was added to the incubation mixture after the incubation, before the extraction steps. The conversion of exogenous [4-^{14}C]cholesterol into 7α-hydroxycholesterol was determined by radioscanning after thin-layer chromatography (8) and the corresponding conversion of endogenous cholesterol was determined by combined gas–liquid chromatography–mass spectrometry as described previously (8). In some experiments, the total conversion of both the endogenous microsomal cholesterol and the exogenous [4-^{14}C]cholesterol was calculated.

Assay of HMG-CoA reductase activity

After preparation of the microsomal fraction as outlined above, incubations with [3-^{14}C]HMG-CoA and subsequent analysis of incubation mixtures were performed essentially as described by Brown, Goldstein, and Dietschy (10). In this assay, the microsomal fraction, 40 μl, is preincubated for 15 min at 37°C in a total volume of 200 μl containing 0.1 M phosphate buffer, pH 7.4, 10 mM imidazole buffer, pH 7.4,5 mM dithiothreitol, 10 mM EDTA, 3 mM NADP, 12 mM glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase. The reaction is then initiated with the addition of 90 nmol (0.5 μCi) of [3-^{14}C]HMG-CoA, dissolved in 25 μl of distilled water, giving a total substrate concentration of 400 μM. The incubation is run for 15 min and stopped by the addition of 25 μl of 6 M HCl. Tritium-labeled mevalonic acid (0.01 μCi) together with 3 mg of unlabeled mevalonic acid lactone is added to the incubation mixture, which is then further lactonized, subjected to thin-layer chromatography, and analyzed for radioactivity.

Assay of microsomal cholesterol and protein

Microsomal free cholesterol was determined by isotope dilution–mass spectrometry after extraction as described previously (11) with the modifications described in ref. 12. The protein concentration was determined according to the method of Lowry et al. (13).

Statistical analysis

The data are expressed as mean ± SEM. The statistical significance of differences was evaluated with the Student's *t*-test.

RESULTS

Apparent saturation of cholesterol 7α-hydroxylase in crude rat liver microsomes

The effect of incubation of rat liver microsomes with varying amounts of unlabeled cholesterol dissolved in 1 mg of Tween 80 on the formation of 7α-hydroxycholesterol is shown in **Table 1**. The product was quantitated by the isotope dilution technique. The apparent saturation of the enzyme was calculated by a comparison between the highest conversion and the conversion obtained in the absence of added cholesterol. As judged from these experiments, the apparent saturation was about 83% for control rats, 75% for starved rats, and 88% for cholesterol-fed rats. Microsomal preparations from cholestyramine-treated rats with high cholesterol 7α-hydroxylase activity had an apparent saturation of cholesterol 7α-hydroxylase of about 84%. The highest degree of conversion always occurred after addition of 75 μg or 150 μg of cholesterol; addition of more than

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TABLE 1. Effect of addition of exogenous substrate on 7α-hydroxylation of cholesterol by liver microsomes from untreated, starved, cholesterol-treated, cholestyramine-treated, and triparanol-treated rats

Addition	Cholesterol 7α-Hydroxylase Activity				
	Untreated Rats (n = 6)	Starved Rats (n = 6)	Cholesterol-Treated Rats (n = 6)	Cholestyramine-Treated Rats (n = 7)	Cholestyramine- and Triparanol-Treated Rats (n = 2)
	pmol/min per mg				
Tween	38 ± 5	16.5 ± 6.8	69 ± 8	103 ± 22	47
Tween + 75 μg of cholesterol	46 ± 4	17.5 ± 7.2	78 ± 3	122 ± 24	
Tween + 150 μg of cholesterol	44 ± 4	22.1 ± 8.8	74 ± 8	123 ± 24	113

The results shown are means ± SEM of experiments with six or seven rats (two rats in the case of treatment with triparanol).

150 μg of cholesterol never did increase the activity (the latter results are not shown).

In separate experiments it was shown that the addition of 1 mg of Tween 80 had no significant effect, or sometimes a slight inhibitory effect (less than 15%), on cholesterol 7α-hydroxylase activity. Addition of more than 2 mg of Tween 80 in general had an inhibitory effect on the activity. Addition of 5 mg of Tween 80 inhibited the reaction by more than 50%. The latter inhibition was possible to overcome by addition of exogenous cholesterol (cf. ref. 14).

All our findings are consistent with a high degree of saturation of cholesterol 7α-hydroxylase in crude rat liver microsomes under most experimental conditions. As demonstrated in previous work (5, 6), all these treatments, with the exception of the cholesterol-feeding, do not significantly affect the total concentration of free cholesterol in the microsomes. In particular, it is noteworthy that the degree of saturation of cholesterol 7α-hydroxylase in microsomes from untreated rats was about the same as that in microsomes from cholesterol-fed rats. The stimulatory effect of dietary cholesterol on cholesterol 7α-hydroxylase activity thus can not be due to an increased saturation of the enzymes.

In order to study whether or not a drastic reduction of the total pools of cholesterol in the body might lead to a reduced degree of saturation of cholesterol 7α-hydroxylase, five rats were treated with cholestyramine (5%) together with a very high dose (0.3% in diet) of the cholesterol synthesis inhibitor triparanol for 4 days. This treatment reduced the serum levels of cholesterol from about 1.6 mmol/l to about 0.6 mmol/l and reduced the concentration of free cholesterol in the microsomes from 25 ± 2 μg/mg of microsomal protein to 11 ± 1 μg/mg of microsomal protein (mean ± SEM). The apparent saturation of cholesterol 7α-hydroxylase was measured in the microsomal preparations from two of these rats and was found to be only about 40% (Table 1). It is thus evident that a drastic reduction of the total concentration of cholesterol in the liver can significantly reduce the substrate saturation of the enzyme.

Experiments with smooth and rough microsomes

The major part of cholesterol 7α-hydroxylase activity is present in the smooth fraction of liver microsomes (15) but some activity can also be found in the rough fraction. Since the smooth and rough fractions have different lipid composition (more neutral lipids in smooth than in rough microsomes (16)), it was considered to be of interest to see whether the degree of substrate saturation of the cholesterol 7α-hydroxylase was different in the two different types of microsomal preparations. The apparent substrate saturation was found to be 70% in smooth microsomes and 91% in rough microsomes prepared from a starved rat.

Experiments with acetone powder

If the degree of substrate saturation of cholesterol 7α-hydroxylase is high under normal conditions, the stimulatory effect of dietary cholesterol of the enzyme cannot be due to increased substrate saturation on the enzyme. The stimulatory effect of the treatment with dietary cholesterol must then be an effect on the enzyme protein and should also be retained after removal of all the cholesterol from the enzyme preparation. That this is the case is evident from the

TABLE 2. Cholesterol 7α-hydroxylase activity in crude and acetone-treated liver microsomes from untreated and cholesterol-fed rats

Source of Microsomal Preparation	Cholesterol 7α-Hydroxylase Activity[a]	
	Microsomes	Acetone Powder
	pmol/min per mg	
Control rats (n = 6)	29 ± 4	3.9 ± 0.8
Cholesterol-fed rats (n = 6)	77 ± 8[b]	9.3 ± 2.0[b]

[a] The activity was measured in the presence of saturating amounts of cholesterol (200 μg) (cf. Experimental Procedure).
[b] Significantly different from control ($P < 0.01$).

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results shown in **Table 2**. After almost complete removal of cholesterol from the microsomes by acetone extraction and subsequent assay of cholesterol 7α-hydroxylase activity in the presence of saturating amounts of substrate (cf. Experimental Procedure), the initial difference between untreated and cholesterol-fed rats was retained. The activity of cholesterol 7α-hydroxylase per mg of protein decreased, however, to less than 20% as a result of the treatment. There is no reason to believe that the degree of inactivation of the enzyme should be different in the two preparations, and the results therefore support the contention that cholesterol availability is not of major importance for the increased 7α-hydroxylase activity obtained after cholesterol feeding.

Effect of treatment with cholesterol-enriched Intralipid and mevalonate on unstimulated and stimulated cholesterol 7α-hydroxylase in rats.

The possibility that HMG-CoA reductase activity per se might be of regulatory importance for cholesterol 7α-hydroxylase under conditions when the enzyme is saturated or almost saturated with substrate was investigated by depression of HMG-CoA reductase by infusion of cholesterol or by treatment with mevalonate. In all these studies, cholesterol 7α-hydroxylase activity was assayed by incubation with a trace amount (10 μg) of [4-^{14}C]cholesterol. The conversion of this labeled cholesterol into 7α-hydroxycholesterol was determined by radioscanning, whereas the conversion of the unlabeled endogenous microsomal cholesterol was determined by isotope dilution–mass spectrometry (5). According to some previous work (17), the pool of endogenous cholesterol available for cholesterol 7α-hydroxylase and for equilibration with exogenous cholesterol may vary under different conditions. Since the enzyme system seems to be saturated or almost saturated with substrate, however, the total conversion of cholesterol into 7α-hydroxycholesterol should best reflect the enzyme activity. It should be pointed out that if the enzyme system is saturated, addition of increasing amounts of exogenous substrate should lead to a decreasing conversion of endogenous substrate.

Intravenous infusion of cholesterol-enriched Intralipid in rats depressed HMG-CoA reductase activity by about 60% as compared to control rats infused with saline (**Table 3**). 7α-Hydroxylation of exogenous cholesterol was unaffected, whereas the corresponding hydroxylation of endogenous and total cholesterol was somewhat increased. The latter increase (about 50%) was, however, not statistically significant ($P > 0.05$).

The above experiment was repeated with bile-fistula rats having activities of HMG-CoA reductase and cholesterol 7α-hydroxylase about 6-fold and 3.5-fold, respectively, higher than non-fistula rats (Table 3). In this case HMG-CoA reductase activity was depressed by about 70%. The 7α-hydroxylation of exogenous, endogenous, and total cholesterol was unaffected or slightly reduced (about 15%) by the Intralipid infusion. The latter reduction was, however, not statistically significant ($P > 0.05$). It is evident that a marked depression of HMG-CoA reductase activity has only small effects on cholesterol 7α-hydroxylation of cholesterol both in the unstimulated and in the stimulated state.

Treatment with cholesterol-enriched Intralipid had little or no effect on the concentration of free cholesterol in the microsomes (effects less than 10%) (Table 3). Treatment with mevalonate in the drinking water was found to depress the HMG-CoA reductase by more than 90% in the unstimulated state and by about 70% in rats stimulated by treatment with cholestyramine (**Table 4**). 7α-Hydroxylation of endogenous and total cholesterol was unaffected by mevalonate treatment, whereas small effects were observed on the 7α-hydroxylation of exogenous cholesterol. Also, in this case, there was little or no effect on the concentration of free cholesterol in the microsomal fraction (Table 4).

TABLE 3. Effect of infusion of cholesterol-enriched Intralipid on HMG-CoA reductase, cholesterol 7α-hydroxylase, and concentration of free microsomal cholesterol in unstimulated rats and bile-fistula rats

Animals	HMG-CoA Reductase Activity	7α-Hydroxylation of			Free Microsomal Cholesterol
		Exogenous Cholesterol	Endogenous Cholesterol	Total Cholesterol	
	nmol/mg per min	*pmol/min per mg*			*μg/mg*
Rats infused with saline (n = 8)	0.44 ± 0.05	7.5 ± 1.4	19.1 ± 2.8	26.6 ± 3.7	17.8 ± 0.9
Rats infused with cholesterol-enriched Intralipid (n = 6)	0.17 ± 0.02[a]	8.0 ± 1.8[b]	29.9 ± 7.3[b]	38 ± 9[b]	19.3 ± 1.2[b]
Bile-fistula rats infused with saline (n = 7)	2.78 ± 0.46	22.8 ± 1.5	64 ± 8	86 ± 9	27 ± 1
Bile-fistula rats infused with cholesterol-enriched Intralipid (n = 6)	0.57 ± 0.16[a]	24.9 ± 3.8[b]	54 ± 4[b]	79 ± 6[b]	26 ± 1[b]

[a] Significantly different from saline control ($P < 0.01$).
[b] Not significantly different from saline control ($P > 0.05$).

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TABLE 4. Effect of treatment with mevalonate (in drinking water) on HMG-CoA reductase, cholesterol 7α-hydroxylase, and concentration of free microsomal cholesterol in unstimulated and cholestyramine-treated rats

Animals	HMG-CoA Reductase Activity	7α-Hydroxylation of Exogenous Cholesterol	7α-Hydroxylation of Endogenous Cholesterol	7α-Hydroxylation of Total Cholesterol	Free Microsomal Cholesterol
	ng/min per mg	*pmol/min per mg*			*μg/mg*
Control rats[a] (n = 6)	0.69 ± 0.11	7.3 ± 0.1	35 ± 2	42 ± 2	23.6 ± 0.9
Rats treated with mevalonate (n = 6)	0.06 ± 0.02[b]	4.7 ± 0.6[b]	31 ± 2[d]	35 ± 2[c]	27.3 ± 1.3[c]
Cholestyramine-treated rats[a] (n = 6)	1.77 ± 0.21	10.9 ± 1.0	64 ± 8	75 ± 8	23.5 ± 1.3
Rats treated with cholestyramine + mevalonate (n = 6)	0.57 ± 0.12[b]	13.7 ± 1.3[c]	67 ± 7[d]	80 ± 6[d]	24.0 ± 2.6[d]

[a] Both the untreated and the cholestyramine-treated control rats were given sucrose, equimolar with the mevalonate.
[b] Significantly different from saline control ($P < 0.01$).
[c] Significantly different from saline control ($P < 0.05$).
[d] Not significantly different from saline control ($P > 0.05$).

It should be noted that the activity of cholesterol 7α-hydroxylase in the control rats, in particular with endogenous cholesterol as substrate, was higher than in the previous experiment, possibly due to the use of sucrose in the drinking water. That treatment with glucose has a stimulatory effect on cholesterol 7α-hydroxylation has been previously documented (18).

The depressive effect of mevalonate on HMG-CoA reductase is very rapid. In order to study the possibility that there may be an initial effect of mevalonate treatment on cholesterol 7α-hydroxylase activity which disappears after some time, mevalonate was administered intravenously to rats; the rats were killed 1 hr later. Also, in these rats there was a marked depression of the HMG-CoA reductase activity as compared to control rats treated with saline (**Table 5**). The mean cholesterol 7α-hydroxylase activity was increased by about 20% as a result of the treatment. This increase was not statistically significant. In similar experiments using a slightly different type of assay, Mitropoulos et al. (2) found a higher degree of stimulation of cholesterol 7α-hydroxylase (70–100%).

DISCUSSION

The most important result of the present study is that the saturation of cholesterol 7α-hydroxylase appeared to be high (70–90%) under most of the experimental conditions employed. This is in accordance with the very low K_m found for a highly purified cholesterol 7α-hydroxylase system from rabbit liver (7). Attempts to determine the actual K_m of the crude microsomal system were never made. The relevance of a K_m for the conversion of a lipophilic substrate by an enzyme system containing a complex mixture of lipids is always questionable. In addition, the determination of the K_m for the microsomal cholesterol 7α-hydroxylase would require removal of the endogenous cholesterol. In our hands such removal of the cholesterol was associated with a considerable loss of enzymatic activity, which, at least in theory, may also affect the K_m.

It is important to point out that a crude microsomal fraction is very heterogeneous, containing membranes and vesicles whose total cholesterol content and ratio of esterified to unesterified cholesterol are different. A sucrose gradient centrifugation of crude microsomes to yield smooth and rough membranes did not, however, yield fractions with widely different degrees of saturation. It may be argued that the addition of a detergent to the complex mixture of different membrane pools in the microsomes may affect the saturation conditions and that the term "saturation" therefore has no independent point of reference. The relatively small amounts of detergent added here had, however, little or no measurable effect on the amount of 7α-hydroxycholesterol formed. The situation in an isolated microsomal fraction is static as compared to the situation in vivo. Even under in vivo conditions, however, with a continuous flux of cholesterol through the system, it seems very unlikely that the cholesterol 7α-hydroxylating system with its low K_m can be unsaturated in a milieu containing high concentrations of substrate. Thus it seems unlikely that substrate availability can be of major importance as a "driving force" for cholesterol 7α-hydroxylase activity.

The present results are in some contrast with those of a previous work by Bosisio, Galli, and Galli (14), who reported that cholesterol 7α-hydroxylase in rat liver microsomes from starved male rats appeared to be unsaturated (below 50%). Starvation of our rats did not significantly change the degree of saturation. Bosisio et al. (14), however, used a concentration of detergent almost 10-fold higher than that used by us. At high concentrations of detergent the endogenous cholesterol may be removed from the microsomes, resulting in falsely low conversion in the absence of added exogenous cholesterol. If the substrate pool of cholesterol is of less importance, the stimulatory effect of cholesterol feeding on cholesterol 7α-hydroxylase activity

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TABLE 5. Effect of intravenous injection of mevalonate on cholesterol 7α-hydroxylase and HMG-CoA reductase

Treatment	HMG-CoA Reductase Activity	7α-Hydroxylation of		
		Exogenous Cholesterol	Endogenous Cholesterol	Total Cholesterol
	nmol/min per mg		*pmol/min per mg*	
NaCl-treated	0.79 ± 0.14	5.1 ± 0.6	34 ± 8	39 ± 8
Mevalonate-treated	0.19 ± 0.03[a]	5.5 ± 0.7[b]	41 ± 7[b]	47 ± 8[b]

[a] Significantly different from saline control ($P < 0.01$).
[b] Not significantly different from saline control ($P > 0.05$).

cannot be due to a simple increase in the degree of saturation of the enzyme. In accordance with this, the stimulatory effect of cholesterol feeding was retained after an almost complete removal of the cholesterol from the microsomes with acetone. This finding is in contrast to a report by Shefer et al. (3). Our results are, however, supported by the finding that further addition of cholesterol to microsomes from untreated rats did not significantly increase the activity, as could have been expected if substrate saturation had been of major importance. The contention that the stimulatory effect of cholesterol feeding on cholesterol 7α-hydroxylase activity is due to activation or induction of enzyme rather than due to substrate availability is also in accordance with our previous finding that treatment with the cholesterol analogue cholestanol leads to an increased 7α-hydroxylation of cholesterol (4). Direct evidence for cholesterol-induced induction of cholesterol 7α-hydroxylase can be obtained first when antibodies towards the cytochrome P-450 component of this enzyme are available.

If the degree of substrate saturation of cholesterol 7α-hydroxylase is high under most experimental conditions, the total concentration of free microsomal cholesterol should be of little or no regulatory importance for the activity. Davis, Musso, and Lattier (19) reported that there was some correlation between the activity of cholesterol 7α-hydroxylase and the cholesterol content of liver microsomes from untreated rats and bile-fistula rats. Using a highly accurate method for cholesterol determination based on isotope dilution–mass spectrometry, however, we found very little variation in the concentration of free microsomal cholesterol under conditions where the activity of the cholesterol 7α-hydroxylase was varied 20-fold (6). In any case, it is evident that even though there may be some correlation between concentration of free microsomal cholesterol and the activity of cholesterol 7α-hydroxylase under some conditions, this cannot be taken as evidence for a regulatory role of the concentration of free microsomal cholesterol.

Our results do not exclude the possibility that substrate saturation may be a limiting factor for cholesterol 7α-hydroxylase in other species and under other experimental conditions. Treatment of rats with very high doses of the cholesterol synthesis inhibitor triparanol resulted in a more than 50% reduction in saturation of cholesterol 7α-hydroxylase. This reduction occurred in parallel with a more than 60% reduction in the serum concentration of cholesterol. Under such extreme conditions, the substrate saturation of cholesterol 7α-hydroxylase must be of importance.

In addition to the degree of saturation of the catalytically active site on cholesterol 7α-hydroxylase, allosteric effects of cholesterol on the enzyme must also be considered. If present, such effects should have been detected with the experimental approach used here.

The mechanism of transfer of cholesterol from its site of synthesis or cellular uptake to cholesterol 7α-hydroxylase is not known. Sterol carrier protein$_2$ (SCP_2) has been shown to have a stimulatory effect on microsomal 7α-hydroxylation of cholesterol (20), but the mechanism of this effect is not known. Thus the possibility has not been excluded that the stimulatory effect is due to removal of an inhibitor or the product (release of product inhibition?). According to the results of the present work, however, it seems unlikely that the mechanism of transport of cholesterol to cholesterol 7α-hydroxylase is of major regulatory importance.

It is evident that, under the conditions employed with a saturated or almost saturated cholesterol 7α-hydroxylase, there is little or no coupling between this enzyme and HMG-CoA reductase. Thus it was possible to depress HMG-CoA reductase markedly with little or no change in cholesterol 7α-hydroxylase activity. This effect was obtained both with unstimulated rats and rats stimulated by biliary drainage or treatment with cholestyramine. These findings are in agreement with a recent study from our laboratory demonstrating that there is little or no coupling between induction of synthesis of HMG-CoA reductase protein and cholesterol 7α-hydroxylase activity under different conditions in rats (5). Thus cholesterol 7α-hydroxylase seems to be regulated independently of HMG-CoA reductase, and the present findings, taken together with previous results from our laboratory (4–6), do not favor the hypothesis that cholesterol synthesis and cholesterol flux are the most important direct regulators of cholesterol 7α-hydroxylase in rat liver.

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REFERENCES

1. Björkhem, I. 1985. Mechanism of bile acid biosynthesis in mammalian liver (review). *In* Comprehensive Biochemistry. H. Danielsson and J. Sjövall, editors. Elsevier Scientific Publ. Co., Amsterdam. 231–278.
2. Mitropoulos, K. A., S. Balasubramaniam, S. Venkatesan, and B. E. A. Reeves. 1978. On the mechanism for the regulation of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase of cholesterol 7α-hydroxylase and of acylcoenzyme A:cholesterol acyltransferase by free cholesterol. *Biochim. Biophys. Acta.* **530:** 99–111.
3. Shefer, S., F. W. Cheng, S. Hauser, A. K. Batta, and G. Salen. 1981. Regulation of bile acid synthesis: measurement of cholesterol 7α-hydroxylase activity in rat liver microsomal preparations in the absence of endogenous cholesterol. *J. Lipid Res.* **22:** 532–536.
4. Björkhem, I., M. S. Buchmann, and S. Skrede. 1985. On the structural specificity in the regulation of the hydroxymethylglutaryl-CoA-reductase and the cholesterol-7α-hydroxylase in rats. Effects of cholestanol feeding. *Biochim. Biophys. Acta.* **835:** 18–22.
5. 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.
6. Einarsson, K., J. E. Åkerlund, and I. Björkhem. 1987. The pool of free cholesterol is not of a major importance for regulation of the cholesterol 7α-hydroxylase activity in rat liver microsomes. *J. Lipid Res.* **28:** 253–256.
7. Boström, H. 1983. Binding of cholesterol to cytochrome P-450 from rabbit liver microsomes. *J. Biol. Chem.* **258:** 15091–15094.
8. Björkhem, I., and A. Kallner. 1976. Hepatic 7α-hydroxylation of cholesterol in ascorbate-deficient and ascorbate-supplemented guinea pigs. *J. Lipid Res.* **17:** 360–365.
9. Bergstrand, A., and G. Dallner. 1969. Isolation of rough and smooth microsomes from rat liver by means of a commercially available centrifuge. *Anal. Biochem.* **29:** 351–356.
10. Brown, M. S., J. L. Goldstein, and J. M. Dietschy. 1979. Active and inactive forms of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver of the rat: comparison with the rate of cholesterol synthesis in different physiological states. *J. Biol. Chem.* **254:** 5144–5149.
11. Björkhem, I., R. Blomstrand, and L. Svensson. 1974. Determination of serum cholesterol by mass fragmentography. *Clin. Chim. Acta.* **54:** 185–193.
12. Schaffer, R., L. T. Sniegoski, M. J. Welch, E. White, A. Cohen, H. S. Hertz, J. Mandel, R. C. Paule, L. Svensson, I. Björkhem, and R. Blomstrand. 1982. Comparison of two isotope dilution–mass spectrometry methods for the determination of total serum cholesterol. *Clin. Chim. Acta.* **28:** 5–8.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
14. Bosisio, E., G. Galli, and M. Galli. 1983. Influence of species and sex on cholesterol 7α-hydroxylase activity in experimental animals. *Eur. J. Biochem.* **136:** 167–172.
15. Mitropoulos, A., S. Venkatesan, S. Balasubramaniam, and T. Peters. 1978. The submitochondrial localization of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, cholesterol 7α-hydroxylase, and cholesterol in rat liver. *Eur. J. Biochem.* **82:** 419–429.
16. Glaumann, H. and G. Dallner. 1968. Lipid composition and turnover of rough and smooth microsomal membranes in rat liver. *J. Lipid Res.* **9:** 720–729.
17. Myant, N. B., and K. A. Mitropoulos. 1977. Cholesterol 7α-hydroxylase. *J. Lipid Res.* **18:** 135–153.
18. Takeuchi, N., M. Ito and Y. Yamamura. 1974. Regulation of cholesterol 7α-hydroxylation by cholesterol synthesis in rat liver. *Atherosclerosis.* **20:** 481–494.
19. Davis, R. A., C. A. Musso, and G. R. Lattier. 1985. Regulation of bile acid biosynthesis by microsomal cholesterol. *In* Enterohepatic Circulation of Bile Acids and Sterol Metabolism. G. Paumgartner, A. Stiehl, and W. Gerok, editors. MTA Press Ltd., Lancaster, England. 37–45.
20. Seltman, H., W. Diven, M. Rizk, B. J. Noland, R. Chanderbhan, T. J. Scallen, G. Vahouny, and A. Sanghvi. 1985. Regulation of bile acid biosynthesis. Role of sterol carrier protein$_2$ in the biosynthesis of 7α-hydroxycholesterol. *Biochem. J.* **230:** 19–24.

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