# On the saturation of the cholesterol 7α-hydroxylase in human liver microsomes

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**Abstract** The relationships between cholesterol 7α-hydroxylase activity, pool of free microsomal cholesterol, and degree of substrate saturation of the enzyme were studied in untreated (n = 5), cholesterol-fed (n = 4), and cholestyramine-treated (n = 6) gallstone patients undergoing cholecystectomy. Highly accurate methods based on isotope dilution–mass spectrometry were used for assay of the cholesterol 7α-hydroxylase activity and for determination of the concentration of free cholesterol in the microsomes. The cholesterol-enriched diet increased the cholesterol 7α-hydroxylase activity about twofold. Cholestyramine treatment was associated with a five- to sixfold increase of the cholesterol 7α-hydroxylase activity. The concentration of free microsomal cholesterol remained essentially unchanged. The apparent degree of saturation of the enzyme was calculated to be 85% in the untreated patients, 86% in the cholesterol-fed patients, and 67% in those treated with cholestyramine. A significant negative correlation was obtained between enzyme activity and apparent substrate saturation. It is concluded that the apparent substrate saturation of the cholesterol 7α-hydroxylase in human liver microsomes is high but that availability of cholesterol may limit the enzyme activity to some extent at high bile acid synthesis rates. —**Einarsson, K., E. Reihnér, and I. Björkhem.** On the saturation of the cholesterol 7α-hydroxylase in human liver microsomes. *J. Lipid Res.* 1989. **30:** 1477–1481.

**Supplementary key words** bile acid synthesis • cholesterol feeding • cholestyramine treatment

The rate-limiting step in bile acid synthesis is catalyzed by the microsomal enzyme cholesterol 7α-hydroxylase. According to several animal experiments, the activity of this enzyme is subject to feed-back inhibition by the bile acids returning to the liver via the portal vein (1, 2). The exact mechanism of the depressing effect of bile acids on the cholesterol 7α-hydroxylase is not known. Davis, Musso, and Lattier (3) have suggested that the size of the substrate pool of cholesterol available for the 7α-hydroxylase is of major regulatory importance. A prerequisite for a regulatory model, in which the substrate pool of cholesterol is of major importance, implies that the cholesterol 7α-hydroxylase is unsaturated with respect to substrate cholesterol. If the enzyme is fully saturated with substrate, changes in the substrate pool should be of little or no importance. However, recent work from our laboratory (4) has indicated a high degree of substrate saturation of the cholesterol 7α-hydroxylase in rat liver microsomes. In accordance with this, there was little or no correlation between the concentration of free cholesterol in the microsomes and cholesterol 7α-hydroxylase activity (5).

Comparatively little is known about the regulation of cholesterol 7α-hydroxylase activity in man. We have recently described an accurate method for assay of the cholesterol 7α-hydroxylase activity in human liver based on isotope dilution–mass spectrometry (6). With use of this assay we have studied the cholesterol 7α-hydroxylase in untreated gallstone patients, patients fed a cholesterol-enriched diet, and patients treated with cholestyramine, a drug known to increase bile acid biosynthesis (7, 8). By addition of exogenous cholesterol to the assay system we have calculated the apparent degree of substrate saturation of the enzyme at both normal and stimulated states of activity. The results show that substrate availability is of little or no regulatory importance for 7α-hydroxylase under normal conditions, but may limit the activity of the enzyme to some extent at high bile acid synthesis rates.

## PATIENTS AND METHODS

### Materials

Deuterium-labeled 7α-hydroxycholesterol was synthesized as described previously (9). Dithiothreitol, EDTA, NADPH, and Tween-80 were purchased from Sigma Chemical Co., St. Louis, MO.

### Patients

Two males and 13 females with gallstone disease, admitted to Huddinge University Hospital for elective cholecystectomy, were included in the study. They were aged 31–64 yr (mean 50), and were all nonobese and normolipidemic. None had clinical or laboratory evidence of

hepatic, intestinal, or renal disease. Four patients were on a cholesterol-enriched diet for 1 week before operation. Six patients were treated with cholestyramine (Questran$^{R}$, Bristol-Myers) in a dose of 8 g b.i.d. for 2–3 weeks prior to operation. All patients gave their informed consent to participate in the study according to the Declaration of Helsinki. The study was approved by the Ethical Committee at Huddinge University Hospital.

### Experimental procedure

Four patients were admitted 1 week before operation to our metabolic unit and were given a cholesterol-enriched diet including five eggs per day corresponding to 3.5 mmol of cholesterol. The other patients were admitted the day before operation. They were given the regular hospital diet containing about 0.5 mmol of cholesterol per day. The operation was performed between 8 and 9 AM after a 12-h fast. A biopsy of 2–4 g was cut out from the left liver lobe, placed in ice-cold buffer, and immediately transported to the laboratory. Cholecystectomy was performed without complications.

### Preparation of liver microsomal fraction

The liver biopsy was minced and homogenized in nine volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM dithiothreitol, and 10 mM EDTA. The homogenate was centrifuged at 20,000 *g* for 15 min. The supernatant fraction was immediately centrifuged at 100,000 *g* for 60 min. The pellet was suspended in homogenizing medium lacking dithiothreitol, and recentrifuged at 100,000 *g* for 60 min. The resulting microsomal fraction was suspended in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, to give a final concentration of 10% (w/v). This microsomal suspension was used for the assay of cholesterol 7α-hydroxylase. The microsomal content of protein was determined by the method of Lowry et al. (10). The concentration of free cholesterol was determined by isotope dilution–mass spectrometry as described previously (11) with a modification as previously described (12).

### Assay of cholesterol 7α-hydroxylase activity

The activity of cholesterol 7α-hydroxylase was assayed as described recently (6). The assay system consisted of 0.5 ml of the microsomal fraction and 0.5 ml 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM NADPH in a total volume of 1.0 ml. After the incubation was stopped, deuterium-labeled 7α-hydroxycholesterol was added as internal standard. The amount of 7α-hydroxycholesterol formed was determined by combined gas–liquid chromatography–mass spectrometry and was expressed as pmol/min per mg protein. In some experiments exogenous unlabeled cholesterol (25 to 130 nmol) was added to the assay. It was dissolved in 100 μg of Tween-80 giving a concentration of 0.01% (w/v) Tween.

### Statistical analysis

The data are expressed as mean ± SEM. The statistical significance of differences was evaluated with the Student's *t*-test. Linear regressions were calculated by the method of least squares, and their significances were tested by estimating the correlation coefficient, *r*.

## RESULTS

In the untreated gallstone patients the cholesterol 7α-hydroxylase activity averaged 7.6 ± 4.0 pmol/min per mg protein and the amount of free cholesterol in the microsomal fraction was 76.6 ± 5.9 nmol/mg protein. The cholesterol-enriched diet increased the enzyme activity in three of four patients, the mean value being about twice as high as in the controls (**Table 1**). Cholestyramine-treated patients had a five- to sixfold higher 7α-hydroxylase activity than the untreated patients (Table 1). The amount of microsomal free choles-

TABLE 1. Cholesterol 7α-hydroxylase activity, concentration of free cholesterol, and apparent degree of saturation of cholesterol 7α-hydroxylase in hepatic microsomes of untreated, cholesterol-fed, and cholestyramine-treated gallstone patients

| Patients | Cholesterol 7α-Hydroxylase Activity | Microsomal Free Cholesterol | Apparent Saturation of Cholesterol 7α-Hydroxylase |
|---|---|---|---|
| | *pmol/min/mg protein* | *nmol/mg protein* | % |
| Untreated (n = 5) | 7.6 ± 4.0 | 76.6 ± 5.9 | 85 ± 6 |
| Cholesterol-fed (n = 4) | 13.4 ± 3.4 | 69.6 ± 14.8 | 86 ± 11 |
| Cholestyramine-treated (n = 6) | 42.3 ± 6.9[a] | 68.4 ± 2.5 | 67 ± 6 |

The results are expressed as mean ± SEM.
[a]Significantly different from untreated patients, $P < 0.005$.

terol was about the same in the three groups of patients (Table 1).

To determine the apparent saturation of the microsomal cholesterol 7α-hydroxylase, varying amounts of unlabeled cholesterol dissolved in Tween-80 were added to the assay system. In separate experiments it was shown that addition of 100 μg of Tween-80 had no significant effect on the cholesterol 7α-hydroxylase activity, whereas addition of as much as 300 μg of Tween-80 inhibited the enzyme activity by about 30%. Addition of 100 μg of Tween-80 caused a slight inhibition of the enzyme activity in only one of the cholestyramine-treated patients. Addition of unlabeled cholesterol up to 130 nmol increased the formation of 7α-hydroxycholesterol by about 15–20% in the untreated patients and by 20–25% in the cholesterol-fed patients (**Table 2**). Addition of corresponding amounts of cholesterol to the microsomal fraction of the cholestyramine-treated patients increased the formation of 7α-hydroxycholesterol by as much as 40% (Table 2). Addition of more than 130 nmol of unlabeled cholesterol did not further increase the conversion.

The apparent saturation of the cholesterol 7α-hydroxylase was defined as 7α-hydroxycholesterol formed in the absence of exogenous cholesterol divided by 7α-hydroxycholesterol formed in the presence of optimal amount of exogenous cholesterol × 100. The coefficient of variation of the apparent saturation, as determined from 14 replicate determinations, was 10 ± 2%. As judged from such calculations the apparent saturation was 85% for untreated patients, 86% for cholesterol-fed patients, and 67% for cholestyramine-treated patients (Table 1). There was a significant negative correlation between the cholesterol 7α-hydroxylase activity and the apparent saturation of the enzyme among the cholestyramine-treated patients ($r = -0.795$, $P<0.01$) and in the combined groups of patients ($r = -0.731$, $P<0.001$) (**Fig. 1**).

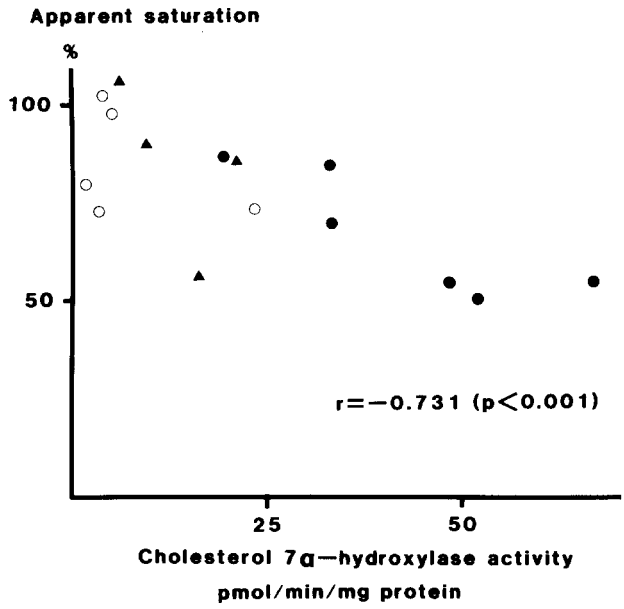


**Fig. 1.** Correlation between cholesterol 7α-hydroxylase activity and apparent saturation of the cholesterol 7α-hydroxylase in liver microsomes of untreated (○), cholesterol-fed (▲), and cholestyramine-treated (●) gallstone patients.

## DISCUSSION

An important finding of the present study was that the apparent saturation of cholesterol 7α-hydroxylase appeared to be as high as 85% in the microsomal fraction of untreated gallstone patients. This result is in agreement with the high saturation of the cholesterol 7α-hydroxylase in rat liver microsomes recently reported (4). Changes in the substrate pool should be of little or no direct regulatory importance for an enzyme highly saturated with substrate. Our finding is also in accordance with a recent work by Boström (13) in which it was

TABLE 2. Effect of addition of exogenous substrate on 7α-hydroxylation of cholesterol by microsomes from untreated, cholesterol-fed, and cholestyramine-treated gallstone patients

| Addition | Cholesterol 7α-Hydroxylase Activity | | |
|---|---|---|---|
| | Untreated Patients (n = 5) | Cholesterol-Fed Patients (n = 4) | Cholestyramine-Treated Patients (n = 6) |
| | *pmol/min/mg protein* | | |
| Tween-80 | 7.6 ± 3.7 | 12.0 ± 3.4 | 33.2 ± 5.7 |
| Tween-80 + 25 nmol of cholesterol | 8.1 ± 4.5 | 14.0 ± 4.4 | 44.9 ± 12.4 |
| Tween-80 + 65 nmol of cholesterol | 9.0 ± 5.3 | 15.4 ± 5.0 | 46.9 ± 8.6[a] |
| Tween-80 + 130 nmol of cholesterol | 8.8 ± 4.5 | 14.2 ± 4.3 | 47.5 ± 10.7[a] |

Cholesterol was added dissolved in 100 μg Tween-80. The results are shown as mean ± SEM.
[a]Significantly different from assays without exogenous cholesterol, $P < 0.05$.

shown that a highly purified cholesterol 7α-hydroxylase system isolated from rabbit liver had a very low $K_m$ (36 μmol/l) towards cholesterol. Since the total concentration of cholesterol in the microsomes is in the millimolar range, a saturated enzyme would be expected from this finding.

It may be argued that it is difficult to determine the substrate saturation when the substrate is insoluble in aqueous medium and is added to the assay system dissolved in a detergent that may affect the microsomal membranes. However, in the present study only small amounts of Tween-80 were used which did not inhibit the enzyme activity.

During the incubation only negligible amounts of the total cholesterol present in the microsomes was converted into 7α-hydroxycholesterol. It is unlikely that the much lower activity present during preparation of the microsomes could have resulted in a significant depletion of substrate cholesterol. It seems even more unlikely that there had been a significant synthesis of cholesterol in the microsomal fraction during the isolation. In view of all this, it seems likely that the substrate saturation of the cholesterol 7α-hydroxylase is similar in vitro and in vivo. As pointed out in a previous study (4), the situation is more static in vitro than in vivo. Also, under in vivo conditions with a continuous flow of cholesterol through the system, it seems likely that the cholesterol 7α-hydroxylase with its low $K_m$ is more or less saturated in a milieu containing high concentrations of substrate.

Cholestyramine treatment stimulated the formation of 7α-hydroxycholesterol five to six times. A cholesterol-enriched diet increased the enzyme activity in three of four patients to a mean value about twice as high as in untreated patients. This observation could not be explained by an increased supply of substrate for the enzyme since the concentration of microsomal free cholesterol was about the same in treated patients as in untreated. It could be argued that crude microsomal fractions such as those used in the present study are very heterogeneous and that the pool of cholesterol available for the cholesterol 7α-hydroxylase may be only a fraction of the total pool of cholesterol in the microsomes. Expansion of a small pool near the enzyme may occur without significant change of the total concentration of free cholesterol in the microsomes. If such an expansion occurs, however, it can hardly be of major regulatory importance in view of the finding that the enzyme was almost saturated with substrate.

The stimulatory effect of cholestyramine treatment as well as cholesterol feeding on the 7α-hydroxylation of cholesterol could thus not be due to an increased degree of substrate saturation of the enzyme. On the contrary, the apparent saturation of the enzyme tended to be lower in the cholestyramine-treated patients compared to the untreated patients. When data from all three groups of patients were combined, a negative correlation was obtained between cholesterol 7α-hydroxylase activity and apparent substrate saturation of the enzyme. From these results it seems likely that availability of cholesterol sometimes may limit the activity of the cholesterol 7α-hydroxylase to some extent in humans. As a consequence we may therefore underestimate the maximal cholesterol 7α-hydroxylating capacity to some extent in patients with high bile acid synthesis rates unless exogenous cholesterol is not added to the assay system. These results are partly different from those obtained in rat experiments. Cholestyramine treatment of rats did not change either the microsomal content of free cholesterol or the degree of saturation of the cholesterol 7α-hydroxylase (4, 5). However, when combining cholestyramine with a potent inhibitor of cholesterol synthesis, the saturation of the cholesterol 7α-hydroxylase decreased significantly also in rats (4).

It is apparent that the increased formation of 7α-hydroxycholesterol in the cholestyramine-treated patients and cholesterol-fed patients must be due to other factors than substrate availability. Whether the stimulatory effect of cholestyramine and cholesterol-feeding on 7α-hydroxylation is due to activation and/or induction of enzyme cannot be decided until antibodies towards the cytochrome P-450 component of the enzyme are available.

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