Bile acid synthesis in man: assay of hepatic microsomal cholesterol 7α-hydroxylase activity by isotope dilution–mass spectrometry

Kurt Einarsson,[1] Bo Angelin, Staffan Ewerth, Klas Nilsell, and Ingemar Björkhem

Departments of Medicine, Surgery, and Clinical Chemistry, Karolinska Institutet at Huddinge University Hospital, Stockholm, Sweden

Abstract The present work describes an accurate assay of the rate-limiting enzyme in bile acid synthesis, the cholesterol 7α-hydroxylase, in human liver. The assay is based on isotope dilution–mass spectrometry, and endogenous microsomal cholesterol is used as the only substrate for the enzyme. Operative liver biopsies were obtained from patients undergoing elective cholecystectomy under highly standardized conditions. In ten gallstone patients, the enzyme activity of the microsomal fraction averaged 9.6 ± 1.4 (mean ± SEM) pmol · min^{-1} · mg protein^{-1} corresponding to a daily synthesis of about 0.5 mmol of bile acids. Three cholestyramine-treated patients displayed a fourfold higher enzyme activity. No evidence was obtained supporting the concept that the cholesterol 7α-hydroxylase is modulated by phosphorylation-dephosphorylation. — **Einarsson, K., B. Angelin, S. Ewerth, K. Nilsell, and I. Björkhem.** Bile acid synthesis in man: assay of hepatic microsomal cholesterol 7α-hydroxylase activity by isotope dilution–mass spectrometry. *J. Lipid Res.* 1986. **27:** 82–88.

Supplementary key words cholestyramine • phosphorylation • dephosphorylation

The first step in the major pathway for conversion of cholesterol into bile acids is 7α-hydroxylation (1, 2). Early studies in the rat gave evidence that this is also the rate-limiting step in the biosynthesis of bile acids (3, 4). The enzyme has been found to be an NADPH-dependent mixed-function oxidase consisting of a specific species of cytochrome P-450 and NADPH-cytochrome P-450 reductase (5, 6). Since 7α-hydroxylation of cholesterol may also occur due to autooxidation or lipid peroxidation, it is difficult to assay the enzyme activity properly. Another complicating factor in the assay is that endogenous microsomal cholesterol serves as substrate and that it appears impossible to assay the activity under conditions of substrate saturation (1, 2). Several approaches have been tried, but the best assays are considered to be those based on measurement of the actual mass of 7α-hydroxycholesterol formed (1, 2), e.g., by isotope dilution–mass spectrometry as described by Björkhem and Danielsson (7).

The occurrence of 7α-hydroxylase activity in human liver was first demonstrated by Björkhem et al. (8), and an enzyme assay based on conversion of labeled substrate was later described by Nicolau et al. (9). The activity in human liver was found to be about one magnitude lower than that in rat liver. This fact, in combination with the difficulty in obtaining sufficient amounts of human liver biopsies, has prevented a more thorough investigation of the properties and regulation of the human enzyme. In view of the great impact of abnormal cholesterol and bile acid metabolism in various human diseases, we considered it important to develop a more accurate method than those previously used for assay of the 7α-hydroxylase activity in human liver. In the present work, such an assay based on isotope dilution–mass spectrometry is described. With use of this assay we have studied the possibility that the cholesterol 7α-hydroxylase is modulated by phosphorylation and dephosphorylation as has been described for the HMG-CoA reductase in human liver (10). We have also determined the activity during treatment with cholestyramine, a drug known to stimulate bile acid biosynthesis in man (11, 12).

MATERIALS AND METHODS

Materials

Deuterium-labeled 7α-hydroxycholesterol was synthesized as described previously (13). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, NADPH, NADH, ATP, EDTA, DTT, and Tween-80 were purchased from Sigma Chemical Co. *E. coli* alkaline phosphatase suspended in 2.6 M ammonium sulfate (30–60 units/mg

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

[1] To whom all correspondence should be addressed at the Department of Medicine, Huddinge University Hospital, S-141 86 Huddinge, Sweden.

protein) was also obtained from Sigma Chemical Co. Just prior to use the enzyme suspension was centrifuged at 12,000 *g* for 45 min at 4°C. The supernatant was discarded and the pellet was suspended in 20 mM imidazole buffer, pH 7.4.

Patients

Thirteen patients admitted to the hospital for elective cholecystectomy were included in the study, two males and eleven females. They were aged 17 to 75 yr, mean age 53 yr. None was overweight or abused alcohol. They had no clinical or laboratory evidence of hepatic or intestinal disease. Three patients had been treated with cholestyramine (Questran^R, Mead-Johnson) in a dose of 8 g twice daily for 3 weeks. None had taken any other drugs known to interfere with lipid metabolism. 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

The patients were admitted to the hospital 2 days prior to operation. They were given the regular hospital diet containing about 0.5 mmol of cholesterol per day. All operations were performed between 8 and 9 AM after a 12-hr fast. After opening of the abdomen a surgical biopsy (1–4 g) was obtained from the left liver lobe. A small piece of the biopsy was sent for histological examination, which in all cases was normal. The other part of the biopsy was immediately placed in ice-cold homogenizing medium and transported to the laboratory within 10 min. After the biopsy was taken a regular cholecystectomy was performed without any complications.

Preparation of liver microsomes

The liver biopsy was minced and homogenized with a loose-fitting Teflon pestle in nine volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM DTT, and 10 mM EDTA. In some experiments, 50 mM NaCl or 50 mM NaF were also included. The homogenate was centrifuged at 20,000 *g* for 15 min. The supernatant solution was centrifuged at 100,000 *g* for 60 min. The microsomal fraction obtained was suspended in a homogenizing medium lacking DTT 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). In experiments with alkaline phosphatase, part of the microsomal fraction was suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose and 1 mM EDTA. The 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. (14).

Assay of cholesterol 7α-hydroxylase activity

The standard assay system consisted of 0.5 ml of the microsomal preparation corresponding to 0.4–1.0 mg of protein, 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM NADPH in a total volume of 1.0 ml. The enzyme assay was carried out in duplicate or triplicate for 15 min at 37°C in air. The reaction was stopped by the addition of 10 ml of chloroform–methanol 2:1 (v/v). Deuterium-labeled 7α-hydroxycholesterol, 150 pmol, dissolved in 25 μl of benzene, was added as internal standard. The chloroform phase was removed and evaporated to dryness under N_2 at about 40°C. The sterol fraction was dissolved in chloroform–methanol 2:1 (v/v) and subjected to thin-layer chromography using benzene–ethyl acetate 1:1 (v/v) as developing solvent. The band corresponding to 7α-hydroxycholesterol and 7β-hydroxycholesterol was eluted with methanol and further analyzed by gas–liquid chromatography–mass spectrometry as described previously (7, 13). Each experiment also included two assays in which the mass of 7α-hydroxycholesterol was determined in the incubation mixture extracted at zero-time. The amount of 7α-hydroxycholesterol formed during an incubation was estimated by subtracting the amount present in the zero-time assay from the amount present at the end of incubation and was expressed as pmol · mg protein^{-1} · min^{-1}. The coefficient of variation of the assay in the normal range of activities, as determined from 54 replicate determinations, was 10%. In a higher range of activities (after cholestyramine treatment) the coefficient of variation was only 4% (6 replicate determinations).

Typical mass fragmentographic recordings obtained in analyses of zero-time and 15-min incubations are shown in **Fig. 1A–C.**

RESULTS

Properties of the cholesterol 7α-hydroxylase assay system

The ratio between 7α-hydroxycholesterol and 7β-hydroxycholesterol, the latter compound being a product of autooxidation of cholesterol, increased several-fold with incubation time, indicating that the 7α-hydroxycholesterol was enzymatically formed. The ratio between 7α-hydroxycholesterol and 7β-hydroxycholesterol was always above 1, and in most incubations no detectable amounts of 7β-hydroxycholesterol were formed. The addition of DTT to the assay system did not affect the formation of 7α-hydroxycholesterol. The rate of formation of 7α-hydroxycholesterol was linear with time for at least 20 min (**Fig. 2**), and an incubation time of 15 min was therefore chosen to assure optimal assay conditions. The rate of

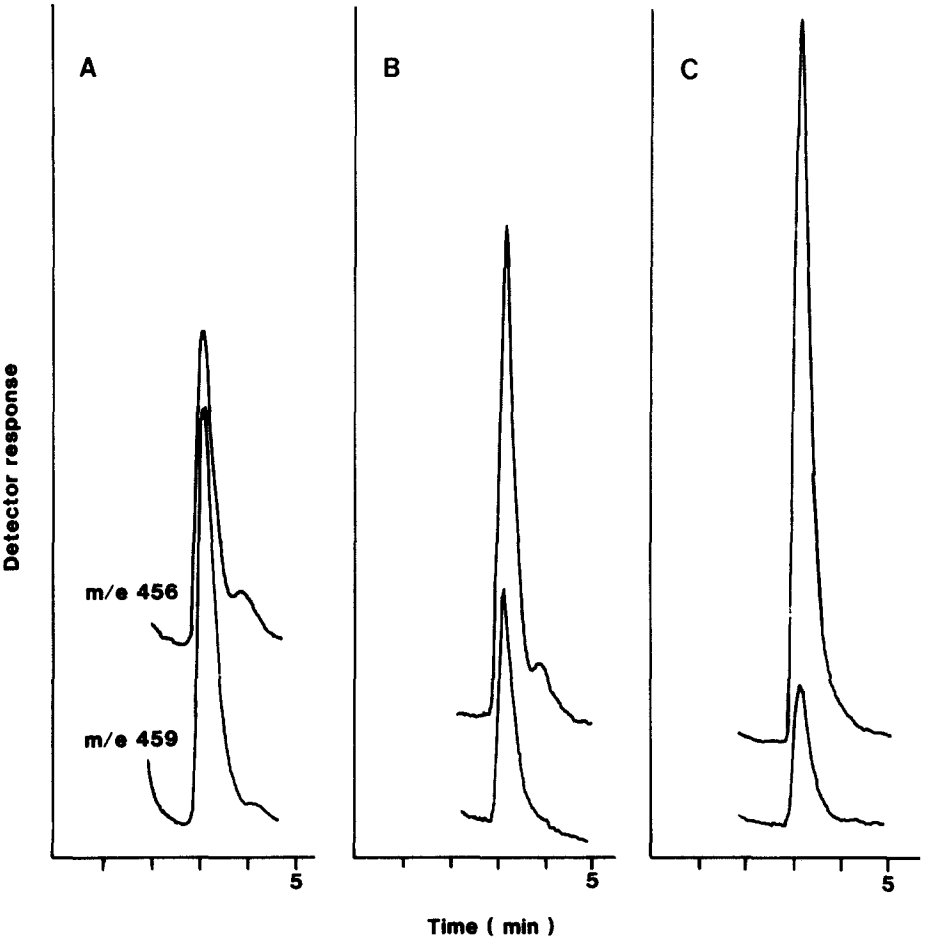

Fig. 1. Selected monitoring of the ions at m/e 456 and m/e 459 of trimethylsilyl ether of purified extract of a zero-time incubation (A), a 15-min incubation of microsomes from liver of an untreated patient (B), and a 15-min incubation of microsomes from liver of a patient treated with cholestyramine (C).

formation of 7α-hydroxycholesterol was proportional to the amount of microsomal fraction added up to about 2 mg of protein per assay (**Fig. 3**). Replacement of NADPH with an NADPH-generating system consisting of NADP, 1 mM, glucose-6-phosphate, 3 mM, and glucose-6-phosphate dehydrogenase, 1 enzyme unit, did not increase the 7α-hydroxylase activity. Addition of NADH, 1 mM, to the NADPH-generating system did not further stimulate the formation of 7α-hydroxycholesterol, as has been described for the rat enzyme (15).

In some experiments, the microsomal fraction was also prepared from very small pieces of a liver biopsy, weighing about 50 mg. In these cases the microsomal fraction added to the assay system contained only 0.10–0.20 mg of protein. However, the 7α-hydroxylase activity obtained was comparable to that obtained in standard assays.

As mentioned above, the cholesterol 7α-hydroxylase utilizes endogenous microsomal cholesterol as substrate and the size of this pool, about 60 nmol · mg protein^{-1}, is probably not sufficient to saturate the enzyme system. Addition of unlabeled cholesterol, up to 130 nmol, with use of Tween-80 increased the total formation of 7α-hydroxycholesterol by about 60% (**Table 1**). Since it has recently been reported that Tween-80 is a noncompetitive inhibitor of the enzyme (16), Tween-80 was also added to the control incubations.

Fig. 2 shows that the formation of 7α-hydroxycholesterol tended to decline after incubation for longer times than about 20 min. A possible explanation is that 7α-hydroxycholesterol was further metabolized. To study this possibility, deuterium-labeled 7α-hydroxycholesterol was added to the incubation mixture at zero time in order to trap the endogenously formed 7α-hydroxycholesterol. Addition of the deuterated compound inhibited the synthesis rate by 50–60% without affecting the time course of the formation of 7α-hydroxycholesterol (Fig. 2).

Attempts to demonstrate reversible activation–inactivation by phosphorylation-dephosphorylation

Tables 2–4 summarize the results of experiments designed to study whether the 7α-hydroxylase may be regulated in vitro by activation–inactivation due to phos-

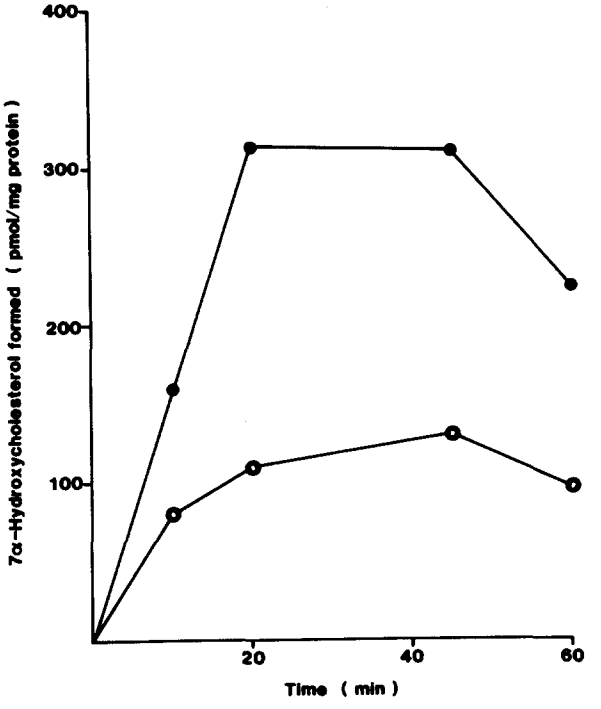

Fig. 2. Effect of incubation time on the cholesterol 7α-hydroxylase activity. ●—●, Standard assay conditions were used; ○—○, deuterium-labeled 7α-hydroxycholesterol was added to the incubation mixture at zero time.

phorylation–dephosphorylation. The presence of 50 mM NaF during homogenization of the liver and preparation of the microsomes did not influence the 7α-hydroxylase activity (**Table 2**). Neither did preincubation of the microsomes with or without 2 mM ATP and 5 mM $MgCl_2$ for 60 min at 37°C have any effect on the enzyme activity (**Table 3**). Preincubation of the microsomal fraction for 60 min at 37°C in 50 mM Tris-HCl buffer containing 0.3 M sucrose and 1 mM EDTA and in the presence of 10 units of *E. coli* alkaline phosphatase did not consistently affect the 7α-hydroxylase activity (**Table 4**). In some incubations a certain degree of stimulation or inhibition was observed, possibly because of unspecific effects of the *E. coli* alkaline phosphatase preparation.

Effect of cholestyramine treatment

The cholesterol 7α-hydroxylase activity averaged 9.6 ± 1.4 (mean ± SEM) pmol · min^{-1} · mg $protein^{-1}$ in ten untreated patients (**Table 5**). In three patients treated with cholestyramine for 3 weeks prior to operation, the enzyme activity averaged 38.5 pmol · min^{-1} · mg $protein^{-1}$, i.e., a fourfold stimulation. The pool size of unesterified microsomal cholesterol, calculated as nmol per mg of protein, was about the same in the cholestyramine-treated patients as in the untreated patients.

DISCUSSION

The development of the present assay of cholesterol 7α-hydroxylase activity in human liver was initiated due to our failure to obtain reproducible results with previously published methods based on conversion of labeled substrate. It should be emphasized that the percentage conversion of labeled cholesterol in the assay by Nicolau et al. (9) is only about 0.1% or less. At this low level of activity, the background level of impurities in the substrate incubated is of great importance and, in our hands, the reproducibility of this assay was insufficient. To our knowledge, the present study presents for the first time an assay system for the human enzyme in which endogenous cholesterol is used as the only substrate for the enzyme. Optimal conditions with regard to incubation time and amount of microsomal fraction were defined. The size of the substrate pool in the microsomal fraction was not sufficient to saturate the enzyme since addition of exogenous cholesterol further increased the amount of 7α-hydroxycholesterol formed. For several reasons it is probably impossible to completely saturate the enzyme by adding exogenous cholesterol (1, 2). It is well known that 7α-hydroxycholesterol as well as other oxidation products of cholesterol may be formed non-enzymatically. Great care was therefore taken to minimize the non-enzymatic oxidation of cholesterol before and after as well as during the incubation procedure. The very small amount of 7β-hydroxycholesterol detected in relation to 7α-hydroxy-

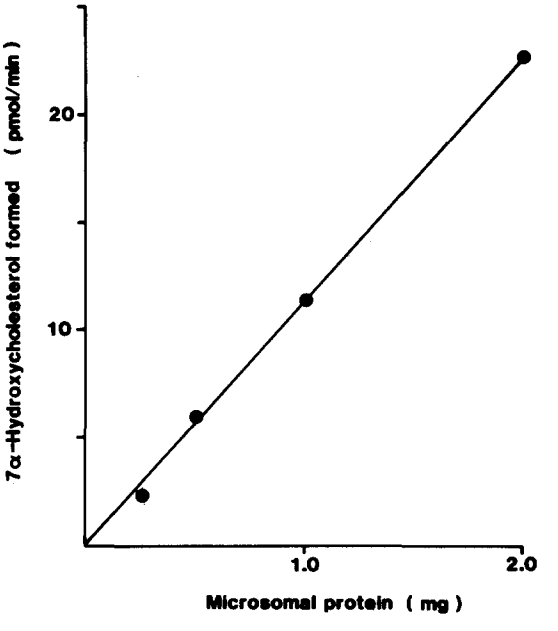

Fig. 3. Correlation between cholesterol 7α-hydroxylase activity and amount of microsomal fraction (mg of protein) added. Standard assay conditions were used.

TABLE 1. Effect of addition of exogenous cholesterol, suspended in 0.3 mg of Tween-80, on hepatic cholesterol 7α-hydroxylase activity

Addition	Relative Enzyme Activity		
	Exp. 1	Exp. 2	Exp. 3
	%		
None (100%)	100	100	100
Tween-80	81	82	51
Cholesterol			
20 nmol		102	68
50 nmol		116	77
65 nmol	130		
130 nmol	133	102	102

cholesterol indicated that autooxidation and lipid peroxidation of cholesterol was kept at a minimum during the experimental conditions used.

Studies in the rat have shown that the activity of the cholesterol 7α-hydroxylase follows a circadian rhythm which is dependent on feeding time and the plasma level of glucocorticoids (1, 2). Whether a circadian rhythm of bile acid synthesis and the cholesterol 7α-hydroxylase also exists in man is not known with certainty (17). Animal studies have further demonstrated that the composition of diet may influence the cholesterol 7α-hydroxylase activity. Thus, in general, addition of cholesterol to the diet has been found to stimulate the enzyme activity (1, 2). Whether this effect is due to an increased amount of enzyme protein or to an expansion of the pool of cholesterol available for the enzyme has not been finally established (18). In order to minimize the effects of diet and potential diurnal variation of the enzyme activity, the patients were given the regular hospital diet 2 days before the operation and the operations were performed between 8 and 9 AM after a 12-hr fast. Anesthesia was also kept standardized. Since it is not known whether the enzyme activity is distributed uniformly throughout the human liver, the liver biopsy was always obtained from the left liver lobe directly after opening the abdomen.

In the present assay, about 0.5 mg of microsomal protein was used. In view of the very high sensitivity in the detection of the trimethylsilyl ether of 7α-hydroxycholesterol (a few picograms may be detected under optimal conditions), it was possible to reduce the size of the biopsy to about 50 mg, which is the size of a liver biopsy obtained percutaneously.

The cholesterol 7α-hydroxylase activity averaged about 10 pmol · min^{-1} · mg $protein^{-1}$ in the untreated subjects. A similar level of cholesterol 7α-hydroxylase activity was found by Salen et al. (19) in the livers of patients with cholesterol gallstones when using the method of Nicolau et al. (9) based on conversion of labeled substrate. Provided that the 7α-hydroxylation of cholesterol is the rate-determining step in the overall synthesis of bile acids in man, this corresponds to a bile acid production of about 0.5 mmol per day. The bile acid synthesis rate in healthy subjects and gallstone patients has previously been determined to be about 1 mmol per day by Lindstedt's isotope dilution technique (20–23). There may be several different explanations for the lower value obtained from our present in vitro data. It is possible that 7α-hydroxylation may increase during day-time because of inflow of dietary cholesterol to the liver, thus expanding the pool of cholesterol available for the enzyme. Another explanation could be that the 7α-hydroxylase activity was not assayed under optimal conditions with respect to access to substrate. Finally, the possibility cannot be completely excluded that bile acids, to a minor degree, may also be synthesized via an alternative pathway not including the 7α-hydroxylation of cholesterol as an initial step, as suggested by Vlahcevic et al. (24).

TABLE 2. Relative cholesterol 7α-hydroxylase activity in human liver microsomes prepared with and without NaF[a]

Source of Enzyme	Relative Enzyme Activity
	%
Microsomes prepared with NaCl, 50 mM	100 ± 12
Microsomes prepared with NaF, 50 mM	93 ± 5

[a]Mean ± SEM of six experiments.

Recently it was suggested that the activity of cholesterol 7α-hydroxylase may be subject to short-term regulation by changes in its phosphorylation state (25–28). Sanghvi et al. (25) and Goodwin, Cooper, and Margolis (28) reported that incubation of rat liver microsomes with *E. coli* alkaline phosphatase produced a loss of cholesterol 7α-hydroxylase activity. Microsomes prepared in the presence of 50 mM NaF had somewhat higher enzyme activity than microsomes prepared with 50 mM NaCl (26, 28). The addition of Mg-ATP could activate or inhibit the 7α-hydroxylase dependent on ATP concentration and pH (25–29). In contrast to these results, we did not find any influence of *E. coli* alkaline phosphatase, NaF, or Mg-ATP on the 7α-hydroxylase activity in human liver microsomes. It should be pointed out that when the human hepatic

TABLE 3. Relative cholesterol 7α-hydroxylase activity in human liver microsomes prepared with and without NaF: effect of preincubation with Mg-ATP[a]

Addition during Preincubation	Microsomes Prepared with NaCl, 50 mM	Microsomes Prepared with NaF, 50 mM
	%	
None	100 ± 22	83 ± 9
Mg, 5 mmol + ATP, 2 mmol	94 ± 5	98 ± 9

[a]Mean ± SEM of six experiments.

TABLE 4. Relative cholesterol 7α-hydroxylase activity in human liver microsomes prepared with and without NaF: effect of preincubation with alkaline phosphatase[a]

Addition during Preincubation	Microsomes Prepared with NaCl, 50 mM	Microsomes Prepared with NaF, 50 mM
	%	
None	100 ± 13	108 ± 18
Alkaline phosphatase, 10 units	98 ± 25	79 ± 11

[a]Mean ± SEM of six experiments.

HMG-CoA reductase activity was studied in our laboratory, under conditions almost identical with those used here, there was significant inhibition by phosphorylation and activation by dephosphorylation (10).

To our knowledge, the present study demonstrates for the first time that cholestyramine treatment stimulates the cholesterol 7α-hydroxylase activity in human liver. Several previous animal studies have shown that interruption of the enterohepatic circulation of bile acids by preparation of a bile fistula or by cholestyramine treatment increases the 7α-hydroxylation of cholesterol (1, 2). Feeding experiments with bile acids have given further evidence that bile acid synthesis and cholesterol 7α-hydroxylase activity are subject to a negative feed-back regulation by bile acids (1, 2). In man, cholestyramine treatment increases bile acid production several-fold (11, 12), whereas cholic acid inhibits chenodeoxycholic acid formation and chenodeoxycholic acid inhibits cholic acid synthesis (22, 30). It thus appears possible that the portal inflow of bile acids to the liver is of importance for the regulation of bile acid synthesis and the activity of the cholesterol 7α-hydroxylase activity in man also. The mechanisms of an effect of bile acids on the cholesterol 7α-hydroxylase are not known. In the present study no evidence was obtained to indicate that the stimulatory effect of cholestyramine treatment on the cholesterol 7α-hydroxylase was due to an increased substrate pool of endogenous microsomal cholesterol. It is more likely that the effect of bile acids may be mediated by changes in the turnover of specific species of cytochrome P-450 with a short half-life, or by modulation of specific proteins in the cytosol (1, 2).

TABLE 5. Effect of cholestyramine treatment on hepatic cholesterol 7α-hydroxylase activity in patients undergoing cholecystectomy

Patient	Treatment	Hepatic Microsomal Cholesterol 7α-Hydroxylase Activity
		pmol · min^{-1} · mg protein^{-1}
1	–	7.7
2	–	11.4
3	–	5.5
4	–	8.1
5	–	21.1
6	–	10.6
7	–	8.2
8	–	7.5
9	–	5.9
10	–	10.2
11	Cholestyramine	31.0
12	Cholestyramine	59.7
13	Cholestyramine	24.7

In conclusion, the present work describes an accurate assay for human cholesterol 7α-hydroxylase activity based on isotope dilution–mass spectrometry, in which endogenous cholesterol is used as the only substrate for the enzyme. Cholestyramine treatment stimulated the enzyme activity several-fold. No evidence for in vitro modulation of enzyme activity by phosphorylation and dephosphorylation could be obtained.

The skillful technical assistance of Ms. Lisbet Benthin and Ms. Gunvor Alvelius and the secretarial help of Ms. Lena Ericsson are gratefully acknowledged. This study was supported by grants from the Swedish Medical Research Council (projects 03X-04793 and 03X-3141) and from the Loo and Hans Osterman Foundation.

Manuscript received 29 May 1984.

REFERENCES

1. Myant, N. B., and K. A. Mitropoulos. 1977. Cholesterol 7α-hydroxylase. *J. Lipid Res.* **18:** 135–153.
2. Björkhem, I. 1985. Mechanism of bile acid biosynthesis in mammalian liver. *In* Comprehensive Biochemistry. Elsevier Scientific Publishing Company, Amsterdam. In press.
3. Danielsson, H., K. Einarsson, and G. Johansson. 1967. Effect of biliary drainage on individual reactions in the conversion of cholesterol to taurocholic acid. *Eur. J. Biochem.* **2:** 44–49.
4. Shefer, S., S. Hauser, J. Berkersky, and E. H. Mosbach. 1970. Biochemical site of regulation of bile acid biosynthesis in the rat. *J. Lipid Res.* **11:** 404–411.
5. Ahlsten, M., R. Hansson, I. Holmberg, I. Ljungdahl, and K. Wikvall. 1981. Reconstitution of cholesterol 7α-hydroxylase system from human liver microsomes. *Biochem. Med.* **26:** 307–313.
6. Erickson, S. K., and B. Bösterling. 1981. Cholesterol 7α-hydroxylase from human liver: partial purification and reconstitution into defined phospholipid-cholesterol vesicles. *J. Lipid Res.* **22:** 872–876.
7. Björkhem, I., and H. Danielsson. 1974. Assay of liver microsomal cholesterol 7α-hydroxylase using deuterated carrier and gas chromatography-mass spectrometry. *Anal. Biochem.* **59:** 508–516.
8. Björkhem, I., H. Danielsson, K. Einarsson, and G. Johansson. 1968. Formation of bile acids in man: conversion of cholesterol into 5β-cholestane-3α,7α,12α-triol in liver homogenates. *J. Clin. Invest.* **47:** 1573–1582.
9. Nicolau, G., S. Shefer, G. Salen, and E. H. Mosbach. 1974. Determination of hepatic cholesterol 7α-hydroxylase activity in man. *J. Lipid Res.* **15:** 146–151.
10. Angelin, B., K. Einarsson, L. Liljeqvist, K. Nilsell and R. A. Heller. 1984. 3-Hydroxy-3-methylglutaryl coenzyme A

reductase in human liver microsomes: active and inactive forms and cross-reactivity with antibody against rat liver enzyme. *J. Lipid Res.* **25:** 1159–1166.
11. Grundy, S. M., E. H. Ahrens, Jr., and G. Salen. 1971. Interruption of the enterohepatic circulation of bile acids in man: comparative effects of cholestyramine and ileal exclusion on cholesterol metabolism. *J. Lab. Clin. Med.* **78:** 94–121.
12. Einarsson, K., K. Hellström, and M. Kallner. 1974. The effect of cholestyramine on the elimination of cholesterol as bile acids in patients with hyperlipoproteinaemia type II and IV. *Eur. J. Clin. Invest.* **4:** 405–410.
13. 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.
14. 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.
15. Björkhem, I., and H. Danielsson. 1973. Heterogeneity of hepatic mixed function oxidases. *Biochem. Biophys. Res. Commun.* **51:** 766–774.
16. Sanghvi, A., E. Grassi, C. Bartman, and W. F. Diven. 1982. Effects of detergents on in vitro 7α-hydroxycholesterol formation by rat liver microsomes. *Lipids.* **17:** 644–649.
17. Duane, W. C., D. G. Levitt, S. M. Mueller, and J. C. Behrens. 1983. Regulation of bile acid synthesis in man. Presence of a diurnal rhythm. *J. Clin. Invest.* **72:** 1930–1936.
18. 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.
19. Salen, G., G. Nicolau, S. Shefer, and E. H. Mosbach. 1975. Hepatic cholesterol metabolism in patients with gallstones. *Gastroenterology* **69:** 676–684.
20. Lindstedt, S. 1957. The turnover of cholic acid in man. *Acta. Physiol. Scand.* **40:** 1–9.
21. Almond, H. R., Z. R. Vlahcevic, C. C. Bell, Jr., D. H. Gregory, and L. Swell. 1973. Bile acid pools, kinetics and biliary lipid composition before and after cholecystectomy. *N. Engl. J. Med.* **289:** 1213–1216.
22. Danzinger, R. G., A. F. Hofmann, J. L. Thistle, and L. J. Schoenfield. 1973. Effect of oral chenodeoxycholic acid on bile acid kinetics and biliary lipid composition in women with cholelithiasis. *J. Clin. Invest.* **52:** 2809–2821.
23. Einarsson, K., K. Hellström, and M. Kallner. 1974. Bile acid kinetics in relation to sex, serum lipids, body weights and gallbladder disease in patients with various types of hyperlipoproteinemia. *J. Clin. Invest.* **54:** 1301–1311.
24. Vlahcevic, Z. R., C. C. Schwartz, J. Gustafsson, L. G. Halloran, H. Danielsson and L. Swell. 1980. Biosynthesis of bile acids in man. Multiple pathways to cholic acid and chenodeoxycholic acid. *J. Biol. Chem.* **255:** 2925–2933.
25. Sanghvi, A., E. Grassi, V. Warty, W. Diven, C. Wight, and R. Lester. 1981. Reversible activation–inactivation of cholesterol 7α-hydroxylase possibly due to phosphorylation–dephosphorylation. *Biochem. Biophys. Res. Commun.* **103:** 886–892.
26. Kwok, C. T., W. Burnett, and I. R. Hardie. 1981. Regulation of rat liver microsomal cholesterol 7α-hydroxylase: presence of a cytosolic activator. *J. Lipid Res.* **22:** 570–579.
27. Kwok, C. T., W. Burnett, and I. R. Hardie. 1981. Regulation of rat liver microsomal cholesterol 7α-hydroxylase: reversible inactivation by ATP + Mg^{2+} and a cytosolic activator. *J. Lipid Res.* **22:** 580–589.
28. Goodwin, C. D., B. W. Cooper, and S. Margolis. 1982. Rat liver cholesterol 7α-hydroxylase. Modulation of enzyme activity by changes in phosphorylation state. *J. Biol. Chem.* **257:** 4469–4472.
29. Kwok, C. T., S. P. Pillay, and I. R. Hardie. 1983. Regulation of rat liver microsomal cholesterol 7α-hydroxylase by Mg ATP: Effect of pH. *Biochem. Biophys. Res. Commun.* **116:** 966–973.
30. Einarsson, K., K. Hellström, and M. Kallner. 1973. Feedback regulation of bile acid formation in man. *Metabolism.* **22:** 1477–1483.