On the possible use of the serum level of 7α-hydroxycholesterol as a marker for increased activity of the cholesterol 7α-hydroxylase in humans

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Abstract The possibility was investigated that the serum level of 7α-hydroxycholesterol can be used as a marker for cholesterol 7α-hydroxylase activity. Six patients with gallstone disease were found to have a mean level of 7α-hydroxycholesterol in serum of 30 ± 4 ng/ml (mean ± SEM) as measured by isotope dilution–mass spectrometry, using deuterated 7α-hydroxycholesterol as internal standard. After treatment with cholestyramine in a dose of 8 g twice daily for 2–3 weeks preoperatively, the serum level increased to 128 ± 20 ng/ml ($P < 0.001$). Eight other patients with gallstone disease had a mean level of 7α-hydroxycholesterol in serum of 29 ± 7 ng/ml. Treatment with chenodeoxycholic acid, 15 mg per kg body weight per day for 3–4 weeks before surgery, decreased the mean level to 20 ± 7 ng/ml ($P > 0.05$). The activity of the cholesterol 7α-hydroxylase in liver biopsies taken during operation was found to be 38 ± 5 pmol/min per mg of protein in the group of patients treated with cholestyramine and 1.3 ± 0.5 pmol/min per mg in the group of patients treated with chenodeoxycholic acid. Liver biopsies from a group of untreated patients (n = 13) had a mean cholesterol 7α-hydroxylase activity of 7.6 ± 1.5 pmol/min per mg. The effect of surgical removal of different lengths of ileum on serum levels of 7α-hydroxycholesterol was studied in patients with ulcerative colitis or morbus Crohn. Removal of 20–100 cm of the terminal part of the ileum led to markedly increased concentrations of 7α-hydroxycholesterol in serum. For patients with a serum level of 7α-hydroxycholesterol above 80 ng/ml (cholestyramine-treated), there was a significant correlation between the serum level of 7α-hydroxycholesterol and the activity of the cholesterol 7α-hydroxylase ($r = 0.59$). It is suggested that serum levels of 7α-hydroxycholesterol may be used for detection of cases with an increased rate of biosynthesis of bile acids. **—Björkhem, I., E. Reihnér, B. Angelin, S. Ewerth, J-E. Åkerlund, and K. Einarsson.** On the possible use of the serum level of 7α-hydroxycholesterol as a marker for increased activity of the cholesterol 7α-hydroxylase in humans. *J. Lipid Res.* 1987. **28:** 889–894.

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Increased flow through a metabolic pathway sometimes leads to an increased leakage of intermediates in this pathway into the circulation. It is well documented that the serum levels of different methyl sterols, intermediates in cholesterol biosynthesis, are increased under conditions of increased biosynthesis of cholesterol (1, 2). In a recent study we showed that there is a good correlation between the concentration of such methyl sterols and the activity of the hepatic HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (Björkhem, I., T. Miettinen, E. Reihnér, B. Angelin, S. Ewerth, and K. Einarsson, unpublished results). In the present study we have investigated the possibility that the serum concentration of 7α-hydroxycholesterol may reflect the activity of the rate-limiting enzyme in bile acid biosynthesis, the hepatic cholesterol 7α-hydroxylase, catalyzing conversion of cholesterol into 7α-hydroxycholesterol. A method for assay of 7α-hydroxycholesterol in serum based on isotope dilution–mass spectrometry was developed. The concentration of 7α-hydroxycholesterol in serum was measured in patients treated with chenodeoxycholic acid and cholestyramine prior to elective cholecystectomy. The activity of the cholesterol 7α-hydroxylase was determined in liver biopsies taken during the operations. A detailed study of the effect of cholestyramine and bile acid feeding on human hepatic 7α-hydroxylase activity will be published elsewhere (Reihnér, E., I. Björkhem, B. Angelin, S. Ewerth, and K. Einarsson, unpublished results).

MATERIALS AND METHODS

Materials

Deuterium-labeled 7α-hydroxycholesterol was synthesized as described previously (3).

Subjects

An initial screening of the patients had been performed at the outpatient clinic. All patients with evidence of diabetes mellitus, hyperlipoproteinemia, or diseases affecting the liver, thyroid and kidney functions had thus been excluded. Informed consent was obtained from each patient before the operation. The study was approved by the Ethical Committee at Huddinge University Hospital. All the patients subjected to surgery are also included in a larger study on the effect of cholestyramine and bile acids on the cholesterol 7α-hydroxylase (Reihnér, E., I. Björkhem, B. Angelin, S. Ewerth, and K. Einarsson, unpublished results).

Healthy control subjects

Five females and three men (ages 23–40 years, mean age 30 years) served as controls.

Subjects treated with chenodeoxycholic acid

Eight females (ages 38–69 years, mean age 55 years) with gallstones received chenodeoxycholic acid, 15 mg/kg body weight per day, for 3–4 weeks before surgery. The blood samples were collected in the morning after a 12-hr fast before the treatment as well as on the day of operation.

Subjects treated with cholestyramine

Six females (ages 41–67 years, mean age 57 years) were treated with cholestyramine (Questran®, Bristol) in a dose of 8 g twice daily 2–3 weeks preoperatively. The blood samples were collected as above.

Untreated patients subjected to surgery

Eleven females and two males (ages 22–77 years, mean age 56 years) with gallstones served as controls to the patients treated with cholestyramine and chenodeoxycholic acid. Blood samples were obtained from only five of these control patients. These samples were collected during the operations and not preoperatively (cf. Results).

Patients subjected to partial resection of ileum

Twelve patients subjected to partial resection of ileum were included in the study. Ten of these patients (ages 24–57 years, mean age 43 years) had colectomy for ulcerative colitis (seven), polyposis coli (two), and Mb Crohn colitis (one) 3 months–18 years previously. Different lengths of the terminal ileum were removed from these patients due to technical operative reasons or due to backwash ileitis (cf. Table 2). Two of the patients were studied on two occasions, first when they had less than 5 cm of the ileum removed and then 3 months after construction of a Park's reservoir and a loop ileostomy with about 100 cm of the terminal ileum excluded.

Two of the patients had Mb Crohn (H, 39 years old and L, 48 years old). Patient H was subjected to resection of different parts of the ileum on three occasions, 1970, 1974, and 1985. In total, about 80 cm of the distal ileum was removed. Patient L was subjected to ileal resection in 1979 and 1983, and in total about 120 cm of the terminal part of the ileum was removed. In 1986 she had a third operation to get a sigmoidestomy. On that occasion a small liver biopsy was taken and part of this biopsy was utilized for assay of cholesterol 7α-hydroxylase activity.

The blood samples from the above patients were collected as above at least 1 month after the resection of the terminal part of the ileum.

Experimental procedure

The patients were hospitalized in the surgical ward 1–2 days before the operation. They were given the regular hospital diet. After a 12-hr fast, the cholecystectomy was performed between 8 AM and 9 AM. After opening of the abdomen, a 2–3-g liver biopsy was cut out from the left liver lobe. A small specimen was sent for histological examination. The rest of the biopsy was immediately placed in ice-cold homogenizing medium and transported to the laboratory within 10 min.

Preparation of liver microsomes

The liver homogenate (10% w/v) was prepared in 50 mM Tris–HCl, pH 2.4, containing 50 mM NaCl, 0.3 M sucrose, 10 mM EDTA, and 10 mM DTT (4). The microsomal fraction was prepared as described previously (4). The microsomal content of protein was determined by the method of Lowry et al. (5).

Assay of cholesterol 7α-hydroxylase activity

Cholesterol 7α-hydroxylase activity in the microsomal fraction was assayed by isotope dilution–mass spectrometry using the same method as previously described (4).

Determination of cholesterol 7α-hydroxycholesterol in serum

In the standard procedure (cf. Results), serum was immediately frozen at −20°C and stored for a maximum of 3 months at that temperature. After thawing, $^2H_3$-labeled 7α-hydroxycholesterol, 150 pmol, dissolved in 130 μl of benzene, was added to 2 ml of serum. Extraction, thin-layer chromatography, and combined gas–liquid chromatography–mass spectrometry were performed exactly as in the assay of cholesterol 7α-hydroxylase activity in human liver microsomes (3, 4). The ion at m/z 456 was used to follow the trimethylsilyl ether derivative of unlabeled 7α-hydroxycholesterol through the chromatography and the ion at m/z 459 was used to follow the $^2H_3$-labeled internal standard (Fig. 1).

In some experiments, butylated hydroxytoluene was added to the serum to give a final concentration of 50 μg of BHT/ml.

RESULTS

Methodology

The method used here for assay of 7α-hydroxycholesterol in serum is essentially the same as that developed in our laboratory for assay of formation of 7α-hydroxycholesterol by microsomal preparations of liver (3, 4, 6). The method involved addition of a deuterated standard to the serum ($^2H_3$-labeled 7α-hydroxycholesterol), extraction, isolation by thin-layer chromatography, derivatization by trimethylsilyl reagent, and analysis by combined gas–liquid chromatography–mass spectrometry. In the latter step the ions at m/z 456 and m/z 459 were followed. From the ratio between the peaks obtained in the two tracings, the amount of unlabeled 7α-hydroxycholesterol can be calculated with use of a standard curve (cf. ref. 6).

In **Fig. 1**, typical tracings are shown, obtained in the analysis of a purified extract of human serum to which $^2H_3$-labeled 7α-hydroxycholesterol had been added. The peak with a retention time at 2.2 min corresponds to the derivative of 7α-hydroxycholesterol, and that at 2.8 min to the derivative of 7β-hydroxycholesterol. In most analyses, the ratio between 7α-hydroxycholesterol and 7β-hydroxycholesterol varied between 2 and 4, but relatively higher peaks of 7α-hydroxycholesterol sometimes occurred.

Under the specific conditions employed, the sensitivity of the assay was 1–2 ng/ml. The precision of the assay was evaluated by replicate measurements of four identical samples of serum from the sample pool, containing 7α-hydroxycholesterol at a concentration of about 14 ng/ml. The coefficient of variation at this low level was found to be 8%. To this pool of serum, 100 ng of 7α-hydroxycholesterol was added per ml. Replicate independent measurements after the addition gave a mean concentration of 119 ± 2 ng/ml (mean ± SD, n = 4). The difference between the expected and observed values was thus 4%. It should be pointed out that the present assay only measures the free form of 7α-hydroxycholesterol, and that a fraction of this compound may be esterified. In order to study this possibility, replicates of the same pool of serum as above were subjected to mild alkaline hydrolysis prior to assay of 7α-hydroxycholesterol. The value obtained after the hydrolysis (14 ± 1 ng/ml; n = 4) was, however, identical to that without hydrolysis, indicating that no significant part of 7α-hydroxycholesterol in serum is esterified under normal conditions. It is known that 7α-hydroxycholesterol, in part, is dehydroxylated during alkaline hydrolysis (7). However, since the deuterated standard was present during the hydrolysis, the standard should have been degraded to the same degree as unlabeled 7α-hydroxycholesterol.

Since the concentration of 7α-hydroxycholesterol in serum was higher than that of 7β-hydroxycholesterol, and 7β-hydroxycholesterol is a major autoxidation product of cholesterol (8), it seems unlikely that the major part of the 7α-hydroxycholesterol in serum can be a product of autoxidation. Prolonged storage as well as repeated freezing and thawing may, however, lead to autoxidation of cholesterol.

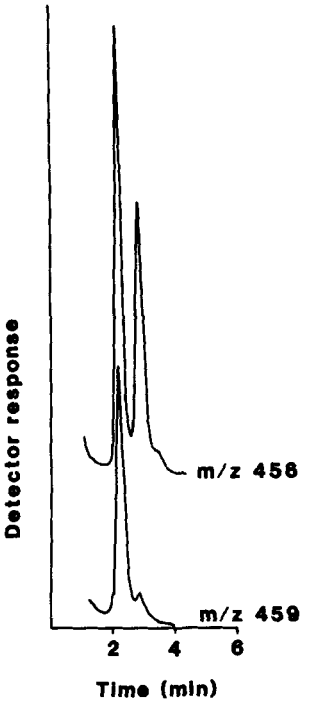

Fig. 1. Selected ion monitoring of the ions at m/z 456 and m/z 459 in analysis of trimethylsilyl ether of a purified extract of serum to which $^2H_3$-labeled 7α-hydroxycholesterol had been added. The peak at about 2.2 min corresponds to 7α-hydroxycholesterol and that at about 2.8 min to 7β-hydroxycholesterol.

In order to study the possible effects of storage, freezing, and thawing on the concentration of 7α-hydroxycholesterol, sera from 12 different individuals were divided into three portions. One portion was extracted and analyzed immediately after the collection, whereas the other two portions were frozen at −20°C in the presence or absence of antioxidant (50 μg of butylated hydroxytoluene per ml of serum). The frozen samples were thawed, extracted, and analyzed 2–3 months later. The samples analyzed immediately were found to have higher levels of 7α-hydroxycholesterol than those subjected to freezing and thawing. The mean level of 7α-hydroxycholesterol was 13 ± 2 ng/ml (mean ± SEM) in the samples analyzed immediately and 8 ± 2 ng/ml and 9 ± 2 ng/ml in the frozen and thawed samples with and without antioxidant, respectively. Freezing and thawing two or more times led to considerably higher levels of 7α-hydroxycholesterol in most cases.

It was concluded that immediate freezing with extraction and analysis within 2–3 months was the optimal proce-

dure. The finding of slightly higher values in samples analyzed immediately could not be explained. In all the subsequent studies, the above optimal procedure was used. With one exception (cf. below) all the samples were collected in the fasting state in the morning (between 7:30 AM and 9:00 AM).

Serum levels of 7α-hydroxycholesterol under conditions of increased and decreased activity of the hepatic cholesterol 7α-hydroxylase

The serum level of 7α-hydroxycholesterol in eight healthy subjects was found to be 23 ± 4 ng/ml (mean ± SEM) when assayed according to the optimal procedure described above.

The six patients treated with cholestyramine (8 g twice daily) were found to have a fasting serum level of 7α-hydroxycholesterol of 30 ± 4 ng/ml (mean ± SEM) prior to the treatment and 128 ± 20 ng/ml after 2–3 weeks of treatment (at the day of operation) (**Table 1**). The difference in serum concentration of 7α-hydroxycholesterol was highly significant ($P < 0.001$, Student's t-test). The activity of the microsomal 7α-hydroxylase in the liver biopsies obtained during the operations was 38 ± 5 pmol/min per mg of protein. The 13 nontreated patients undergoing cholecystectomy had a cholesterol 7α-hydroxylase activity of 7.6 ± 1.5 pmol/min per mg of protein, and it may thus be concluded that the cholestyramine treatment had increased the activity of the cholesterol 7α-hydroxylase about fivefold.

The eight patients treated with chenodeoxycholic acid (15 mg/kg body weight per day) were found to have fasting serum levels of 7α-hydroxycholesterol of 29 ± 7 ng/ml prior to the treatment and 20 ± 7 ng/ml after 3–4 weeks on the treatment (a few days prior to operation). The difference in serum concentration of 7α-hydroxycholesterol was not significant when evaluated with the paired or unpaired t-test ($P > 0.05$). The activity of the microsomal 7α-hydroxylase in the liver biopsies obtained during the operation was 1.3 ± 0.5 pmol/min per mg, which is significantly lower than the corresponding activity found in the 13 untreated patients (Table 1).

Blood samples were obtained from only 5 of the 13 untreated gallstone patients subjected to cholecystectomy. Since these blood samples were taken during the operation and not preoperatively, the results are not directly comparable. The mean concentration of 7α-hydroxycholesterol in serum from the 5 untreated patients was 12 ± 2 ng/ml. This value is considerably lower than that obtained from the healthy subjects and from the patients treated with cholestyramine and chenodeoxycholic acid prior to the treatment. The lower level of 7α-hydroxycholesterol may be due to the premedication or some other factor related to the operation.

There was no significant correlation between the activity of the hepatic cholesterol 7α-hydroxylase and the concentration of 7α-hydroxycholesterol in serum in the untreated patients and the patients treated with chenodeoxycholic acid. The concentration of 7α-hydroxycholesterol ranged from 0 to 61 ng/ml in these 13 patients. There was, however, a significant correlation between the two parameters in the case of the patients treated with cholestyramine ($r = 0.59$). The serum concentration of 7α-hydroxycholesterol ranged from 82 ng/ml to 208 ng/ml in these patients.

The correlation between the activity of the hepatic cholesterol 7α-hydroxylase activity and the serum level of 7α-hydroxycholesterol in the patients treated with cholestyramine and chenodeoxycholic acid is shown in **Fig. 2.**

Serum levels of 7α-hydroxycholesterol in patients subjected to removal of various lengths of the terminal ileum

Table 2 summarizes the levels of 7α-hydroxycholesterol in patients in which different lengths of the terminal ileum had been removed. Normal or only slightly elevated concentrations of 7α-hydroxycholesterol in serum were obtained in patients subjected to removal of less than 20 cm of the terminal ileum. Removal of 20 cm (patient G)

TABLE 1. Serum levels of 7α-hydroxycholesterol and activity of the hepatic microsomal 7α-hydroxylase in the three different groups of patients[a]

Group of Patients	Serum Levels of 7α-Hydroxycholesterol prior to Treatment	Serum Levels of 7α-Hydroxycholesterol on the Day of Operation	Activity of the Cholesterol 7α-Hydroxylase in the Liver Biopsy Taken during the Operation[b]
	ng/ml		*pmol/min per mg protein*
Treated with cholestyramine (n = 6)	30 ± 4	128 ± 20	38 ± 5
Treated with chenodeoxycholic acid (n = 8)	29 ± 7	20 ± 7	1.3 ± 0.5
Untreated (n = 13)			7.6 ± 1.5

[a] The values given are means ± SEM.

[b] The liver biopsies were some of those utilized in a more extensive study on the effect of cholestyramine and chenodeoxycholic acid on cholesterol 7α-hydroxylase activity in human liver (Reihnér, E., I. Björkhem, B. Angelin, S. Ewerth, and K. Einarsson, unpublished results). Serum was not available from all the patients involved in the latter study.

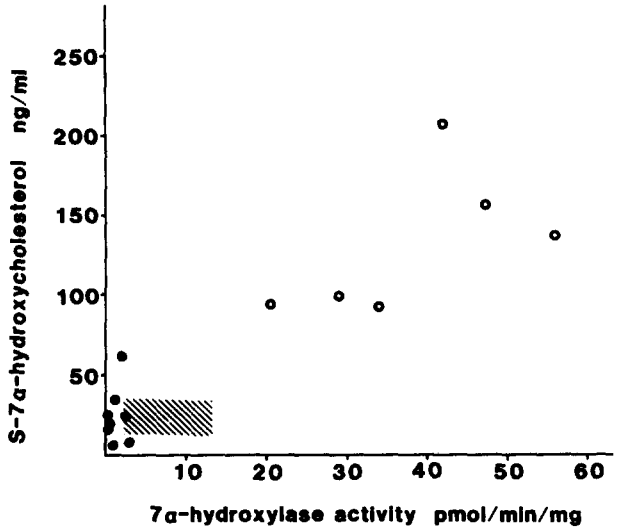

Fig. 2. Correlation between the cholesterol 7α-hydroxylase activity and the serum level of 7α-hydroxycholesterol in patients treated with chenodeoxycholic acid (●) and cholestyramine (○). The hatched area in the figure corresponds to the normal range (mean ± 1 SD), obtained by measurement of cholesterol 7α-hydroxylase activity in 13 untreated patients with gallstone disease (Table 1) and serum level of 7α-hydroxycholesterol in 8 healthy untreated subjects (see text).

or more (patients H, I, J, K, and L) led to markedly elevated levels, 61–183 ng/ml.

Blood samples were obtained from two of the patients (J and K) prior to an operation in which about 100 cm of the terminal ileum was removed (cf. Materials and Methods). The levels of 7α-hydroxycholesterol in serum were only slightly elevated prior to the operation (possibly due to diarrhea), but markedly elevated 1 month or more after the operation.

It was possible to obtain a liver biopsy from one of the patients (L) about 3 years after the resection of about 120 cm of the terminal part of the ileum. As expected, the activity of the cholesterol 7α-hydroxylase was markedly high, about 41 pmol/min per mg protein. This level is similar to that obtained in the subjects treated with cholestyramine (Table 1 and Fig. 2).

DISCUSSION

As far as known, enzymatic formation of 7α-hydroxycholesterol occurs exclusively in the liver, and from the results obtained, it seems likely that a major part of the 7α-hydrocholesterol present in serum is derived from the liver. When the activity of the hepatic cholesterol 7α-hydroxylase was normal or depressed, however, there was little or no correlation between this activity and the level of 7α-hydroxycholesterol in serum. Treatment with chenodeoxycholic acid, which is known to depress the cholesterol 7α-hydroxylase activity markedly in human liver microsomes (Reihnér, E., I. Björkhem, B. Angelin, S. Ewerth, and K. Einarsson, unpublished results), had a small effect on the level of 7α-hydroxycholesterol in serum. The relatively wide range of concentrations of 7α-hydroxycholesterol in serum of untreated patients may in part be due to the presence of a small and variable extent of autoxidation of cholesterol which may produce different amounts of 7α-hydroxycholesterol. On the other hand, all the serum samples from the patients treated with cholestyramine had markedly increased levels of 7α-hydroxycholesterol in serum (above 80 ng/ml), and in this case there was a significant correlation between the serum level of 7α-hydroxycholesterol and the activity of the cholesterol 7α-hydroxylase. A significant leakage of 7α-hydroxycholesterol into the circulation thus seems to occur first when the activity of the cholesterol 7α-hydroxylase is increased above a certain level. Under such conditions, the variable fraction of 7α-hydroxycholesterol originating

TABLE 2. Effect of partial resection of ileum on serum levels of 7α-hydroxycholesterol

Patient	Sex	Age	Diagnosis[a]	Length of Resected Part of Distal Ileum	Concentration of 7α-Hydroxycholesterol	
					Prior to Operation	After Operation
		yr		*cm*	*ng/ml*	
A	F	38	uc	5		8
B	F	57	cp	5		8
C	F	48	uc	5		12
D	M	44	uc	15–17		21
E	F	38	mC	16–17		39
F	M	47	cp	17		28
G	M	43	uc	20		91
H	F	39	mC	80		61
I	M	24	uc	100		94
J	M	34	uc	100	20	107
K	M	56	uc	100	37	183
L	F	48	mC	120		110

[a] uc, ulcerative colitis; cp, colonic polyposis; mC, morbus Crohn.

from other sources than the hepatic cholesterol 7α-hydroxylase will be less important.

A leakage of enzymatically formed 7α-hydroxycholesterol from the liver into the circulation may appear surprising in view of the fact that the cholesterol 7α-hydroxylase is rate-limiting in the overall biosynthesis of bile acids from cholesterol. The activity of the cholesterol 7α-hydroxylase is thus considerably lower than the activity of the other enzymes involved in the biosynthesis. Under optimal in vitro conditions, human liver microsomes can oxidize 7α-hydroxycholesterol into 7α-hydroxy-4-cholesten-3-one at a rate about 1000-fold higher than the rate of 7α-hydroxylation of cholesterol (4, 9). On the other hand, the K_m of the 3-hydroxysteroid dehydrogenase active on 7α-hydroxycholesterol seems to be relatively high (a K_m of 0.5×10^{-4} M has been reported (10) for a purified enzyme preparation from rabbit liver) and a certain accumulation of 7α-hydroxycholesterol is therefore possible. In accordance with this, we have previously shown that the concentration of 7α-hydroxycholesterol in human liver microsomes is considerably higher than the concentration of other intermediates in bile acid biosynthesis such as 7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α-diol, and 5β-cholestane-3α,7α,12α-triol (11). It cannot be excluded that the accumulated 7α-hydroxycholesterol may be of some regulatory importance, since it has been reported that there is some product inhibition of the cholesterol 7α-hydroxylase (12). In any case, the finding that there are relatively high concentrations of 7α-hydroxycholesterol in liver microsomes supports the contention that a small leakage can occur from the liver into the circulation.

The finding that an increased activity of the cholesterol 7α-hydroxylase is associated with elevated levels of 7α-hydroxycholesterol in serum may be of some diagnostic interest. It was convincingly shown that patients with malabsorption due to surgical removal of the terminal part of the ileum had markedly increased levels of 7α-hydroxycholesterol in serum, provided the length of the removed part was about 20 cm or more. From unpublished studies in our laboratory, we know that such patients have levels of hepatic cholesterol 7α-hydroxylase similar to those of patients treated with cholestyramine. This was confirmed in one of the patients studied here from whom a liver biopsy was obtained about 3 years after removal of about 120 cm of the distal part of the ileum. The possibility that determination of serum levels of 7α-hydroxycholesterol can be used as a routine method for detection of cases with an increased rate of biosynthesis of bile acids is at present under study.

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