Effect of biliary obstruction on 26-hydroxylation of C_{27}-steroids in bile acid synthesis

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Abstract The effect of biliary obstruction on side chain hydroxylations in the biosynthesis and metabolism of bile acids was studied in the rat. For comparison, several other hydroxylation reactions in bile acid biosynthesis and metabolism were assayed. Biliary obstruction inhibited microsomal 26-hydroxylation of 5β-cholestane-3α,7α-diol and microsomal 25- and 26-hydroxylation of 5β-cholestane-3α,7α-12α-triol. Microsomal 7α-hydroxylation of cholesterol and 6β-hydroxylation of lithocholic acid increased significantly, whereas the increase in microsomal 12α-hydroxylation of 5β-cholestane-3α,7α-diol was less. Mitochondrial 26-hydroxylation of cholesterol, 5-cholestene-3β,7α-diol, and 7α-hydroxy-4-cholesten-3-one was stimulated, whereas 26-hydroxylation of 5β-cholestane-3α,7α-diol was not affected and that of 5β-cholestane-3α,7α,12α-triol was markedly inhibited. The results indicate that mitochondrial 26-hydroxylation, particularly of substrates that primarily are precursors of chenodeoxycholic acid, plays a more important role in bile acid biosynthesis under conditions of biliary obstruction than under normal conditions.

Supplementary key words side chain hydroxylations

Biliary obstruction leads to a marked change in the pattern of bile acid biosynthesis in the rat (1). β-Muricholic acid becomes a predominant bile acid and the amount of cholic acid synthesized is reduced drastically. Evidence has been presented to indicate that total bile acid biosynthesis decreases in biliary obstruction (2). A previous report from this laboratory showed that the increase in β-muricholic acid synthesis in biliary obstruction is reflected in an increase in microsomal 6β-hydroxylation of taurochenodeoxycholic acid and lithocholic acid (3). In the same report, several other hydroxylations in the biosynthesis and metabolism of bile acids were assayed. The rate of 7α-hydroxylation of cholesterol, the main rate-limiting step in bile acid biosynthesis, was found to increase markedly in spite of the apparent decrease in overall bile acid biosynthesis in biliary obstruction (3).

The aim of the present work was to further define mechanisms responsible for the change in pattern of bile acid biosynthesis under conditions of biliary obstruction and thereby obtain information concerning the mechanisms that govern the proportion of cholic acid and chenodeoxycholic acid synthesized from cholesterol. Mitochondrial and microsomal 26-hydroxylation of a number of C_{27}-steroids has been assayed in rats with ligated bile duct. For comparison, several other hydroxylations in bile acid biosynthesis and metabolism have been analyzed.

METHODS

Materials

[4-^{14}C]Cholesterol (60 μCi/μmol) was obtained from Radiochemical Centre, Amersham, England. Before use, the material was purified by chromatography on a column of aluminum oxide, grade III (Woelm, Eschwege, Germany). 5-[7β-^{3}H]Cholestene-3β-7α-diol (6 μCi/μmol), 7α-hydroxy-4-[6β-^{3}H]cholesten-3-one (7 μCi/μmol), 5β-[7β-^{3}H]cholestane-3α,-7α-diol (7 μCi/μmol), and 5β-[7β-^{3}H]cholestane-3α,-7α,12α-triol (7 μCi/μmol) were prepared as described previously (4). [24-^{14}C]Lithocholic acid (1.5 μCi/μmol) was obtained from NEN Chemicals, Dreieichenhain, Germany. (25*R*)5-Cholestene-3β,26-diol, 5-cholestene-3β,7α,26-triol, 5β-cholestane-3α,7α,26-triol, and 5β-cholestane-3α,7α,12α,26-tetrol were prepared as described previously (4). NADPH was obtained from Sigma Chemical Co., St. Louis, MO.

Methods

Male rats of the Sprague-Dawley strain weighing about 200 g were used. The animals were fed a rat chow diet (Anticimex, Stockholm, Sweden) ad libitum.

Abbreviations: The following systematic names are given to bile acids referred to by trivial names: cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid.

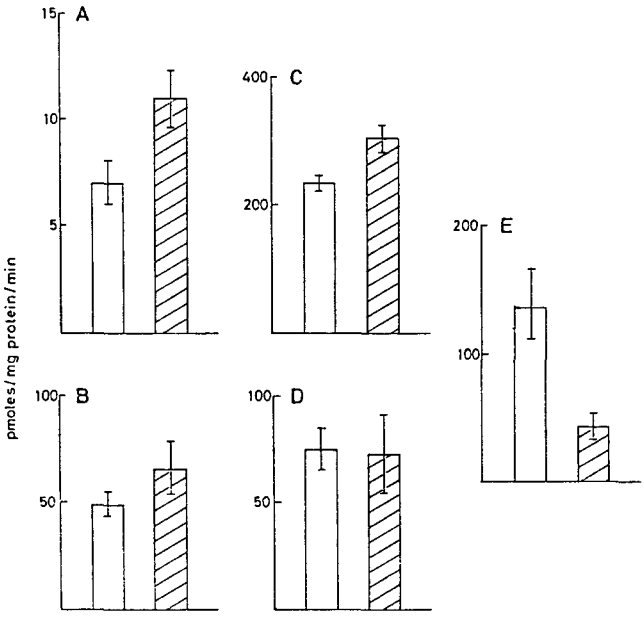

Fig. 1. Effect of biliary obstruction on mitochondrial 26-hydroxylation of cholesterol (*A*), 5-cholestene-3β,7α-diol (*B*), 7α-hydroxy-4-cholesten-3-one (*C*), 5β-cholestane-3α,7α-diol (*D*), and 5β-cholestane-3α,7α,12α-triol (*E*). The values are the means ± SE of the means of results obtained with four rats. Open bars, control rats; cross-hatched bars, obstructed rats.

The common bile duct was ligated proximally and distally under ether anesthesia. As controls, sham-operated rats were used. The rats were killed 96 hr after operation. Liver homogenates, 20% (w/v), were prepared in 0.25 M sucrose containing 1 mM EDTA when the microsomal fraction was isolated (3). The homogenate was centrifuged at 20,000 *g* for 15 min. The microsomal fraction was obtained by centrifuging the 20,000 *g* supernatant fluid for 70 min at 100,000 *g*. The microsomal fraction was suspended in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.028 M nicotinamide in the case of incubations with [4-^{14}C]cholesterol and [24-^{14}C]lithocholic acid (3) and in 0.1 M Tris-Cl buffer, pH 7.4, in the case of incubations with 5β-[7β-^{3}H]cholestane-3α,7α-diol and 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (4). The mitochondrial fraction was prepared as described by Sottocasa et al. (5). Liver homogenate, 10% (w/v), was centrifuged at 600 *g* for 15 min and the 600 *g* supernatant was centrifuged at 6,500 *g* for 20 min. The pellet was washed twice with one-half and one-fourth of the initial volume of sucrose. The mitochondrial fraction was finally suspended in 0.1 M Tris-Cl buffer, pH 7.4, in a volume corresponding to the volume before the last centrifugation. Membrane-disintegrated mitochondria were prepared by the swelling–condensing–sonication method described by Sottocasa et al. (5). The incubations were carried out at 37°C. [4-^{14}C]Cholesterol, 0.03 μmol in a suspension with Tween 80, was incubated for 15 min with 3 ml of microsomal fraction and 3 μmol of NADPH in a total volume of 5 ml (6). The Tween 80 suspension was prepared by dissolving the appropriate amount of [4-^{14}C]cholesterol in 0.1 ml of methanol and adding 3 mg of Tween 80 dissolved in 0.1 ml of methanol. The methanol was evaporated under a stream of nitrogen and 0.1 ml of buffer was added (6). [24-^{14}C]Lithocholic acid, 0.2 μmol in 50 μl of acetone, was incubated for 20 min with 1 ml of microsomal suspension and 3 μmol of NADPH in a final volume of 3 ml (3). 5β-[7β-^{3}H]Cholestane-3α,7α-diol and 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol, 0.63 μmol in 50 μl of acetone, were incubated with 1.5 ml of microsomal suspension in a total volume of 3 ml. An NADPH-generating system was used (4). In incubations with the mitochondrial fraction, 0.25 μmol of [4-^{14}C]cholesterol, 5-[-7β-^{3}H]cholestene-3β,7α-diol, 7α-hydroxy-4-[6β-^{3}H]cholesten-3-one, 5β-[7β-^{3}H]cholestane-3α,7α-diol, and 5β-[7β-^{3}H]-cholestane-3α,7α,12α-triol were incubated for 40 min with 1 ml of mitochondrial fraction and 4.6 μmol of isocitrate in a total volume of 3 ml (7). In incubations with [4-^{14}C]cholesterol, Mg^{2+} was added to a final concentration of 10 mM. Incubations with all substrates except lithocholic acid were terminated by the addition of 20 volumes of chloroform–methanol 2:1 (v/v) (4). After addition of 0.2 volumes of 0.9% (w/v) sodium chloride solution, the chloroform phase was collected and the solvent was evaporated. The residue was subjected to thin-layer chromatography. In some cases, the corresponding 26-hydroxylated compounds were added as internal standards. Incubations with lithocholic acid were terminated by the addition of 5 ml of 95% (v/v) aqueous ethanol (3). After dilution with water and acidification, the mixture was extracted two times with ether. The combined ether extracts were washed with water until neutral and the solvent was evaporated.

The following solvent mixtures were used for thin-layer chromatography: benzene–ethyl acetate 2:3 (v/v) for incubations of cholesterol with microsomes (6); benezene–ethyl acetate 1:1 (v/v) for incubations of cholesterol with mitochondria (4); ethyl acetate for incubations with 5-cholestene-3β,7α-diol and 5β-cholestane-3α,7α-diol; benzene–ethyl acetate 1:4 (v/v) for incubations with 7α-hydroxy-4-cholesten-3-one (4); and system S 11 (3) for incubations with 5β-cholestane-3α,7α,12α-triol and lithocholic acid. The extent of conversion of substrate was calculated from radio scans of the thin-layer chromatograms with a thin-layer scanner (Berthold, Karlsruhe, Germany). The 26-hydroxylated products were further identified by radio–gas–liquid chromatography of the tri-

methylsilyl ether of the material eluted from the thin-layer plates. A 1% SE-30 column was used in a Packard radio–gas chromatograph. With this method, 25- and 26-hydroxylated products are separated completely. This method was used after thin-layer chromatography in analyses of 25- and 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol. Conversions of cholesterol in mitochondrial as well as microsomal preparations were measured on the basis of the exogenous cholesterol added. The content of cholesterol, measured by gas–liquid chromatography of the trimethylsilyl ether derivative on a column of 3% QF-1 or 1% SE-30, was on an average 18 μg (14–19 μg) per ml of mitochondrial suspension from control rats, 21 μg (15–24 μg) per ml of mitochondrial suspension from obstructed rats, 100 μg (72–114 μg) per ml of microsomal suspension from control rats, and 93 μg (77–108 μg) per ml of microsomal suspension from obstructed rats. Protein was determined according to Lowry et al. (8).

RESULTS

Mitochondrial hydroxylations

Fig. 1 shows the effect of biliary obstruction on mitochondrial 26-hydroxylation of cholesterol (*A*), 5-cholestene-3β,7α-diol (*B*), 7α-hydroxy-4-cholesten-3-one (*C*), 5β-cholestane-3α,7α-diol (*D*), and 5β-cholestane-3α,7α,12α-triol (*E*). In the group of experiments shown in Fig. 1, the extent of stimulation of 26-hydroxylation of cholesterol was about 50%. In other experiments, stimulations of up to about 150% were found (cf. Table 1). 26-Hydroxylation of cholesterol by the mitochondrial fraction is always accompanied by a 25-hydroxylation which is about one-tenth of the 26-hydroxylation (9). Although the extent of 25-hydroxylation was not determined accurately, gas–liquid chromatographic data showed a similar proportion between 26- and 25-hydroxylation both in the rats with biliary obstruction and in the sham-operated rats. 26-Hydroxylation of 5-cholestene-3β,7α-diol and 7α-hydroxy-4-cholesten-3-one were stimulated about 30% by biliary obstruction, whereas 26-hydroxylation of 5β-cholestane-3α,-7α-diol was not affected. The 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol was inhibited by about 70%. No significant formation of C_{27}-acid was detected in the incubations of 5β-cholestane-3α,7α,12α-triol (cf. ref. 4).

There was no marked difference in effect of substrate concentration on reaction rates between control rats and rats with biliary obstruction. In **Fig. 2** is shown the effect of substrate concentration on

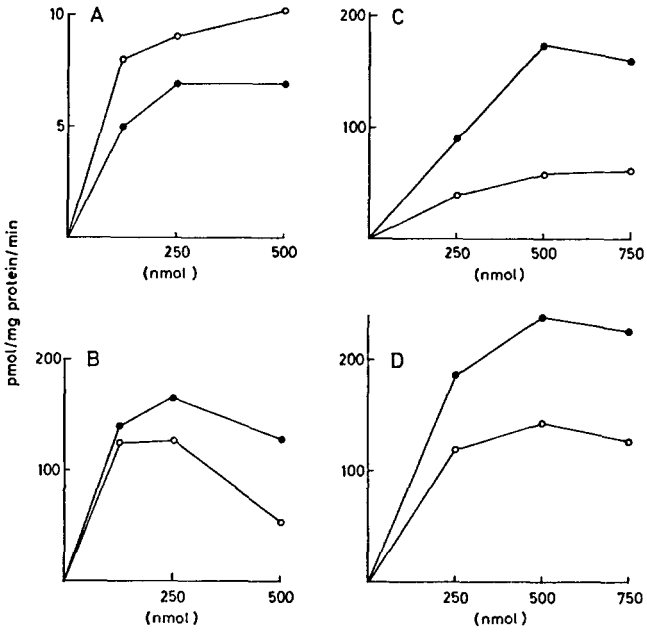

Fig. 2. Effect of substrate concentration on mitochondrial and microsomal side chain hydroxylations. The diagrams show mitochondrial 26-hydroxylation of cholesterol (*A*), mitochondrial 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (*B*), microsomal 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol (*C*), and microsomal 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (*D*). Symbols: ●, control rats; ○, obstructed rats.

the two mitochondrial reactions primarily affected by biliary obstruction, 26-hydroxylation of cholesterol and 5β-cholestane-3α,7α,12α-triol.

Microsomal hydroxylations

Fig. 3 shows the effect of biliary obstruction on microsomal 7α-hydroxylation of cholesterol (*A*), 12α-hydroxylation of 5β-cholestane-3α,7α-diol (*B*), 26-hydroxylation of 5β-cholestane-3α,7α-diol (*C*), 25- and 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (*D* and *E*), and 6β-hydroxylation of lithocholic acid (*F*). In agreement with previous work (3), biliary obstruction was found to stimulate microsomal 7α-hydroxylation of cholesterol three- to fourfold and 6β-hydroxylation of lithocholic acid about twofold. 12α-Hydroxylation of 5β-cholestane-3α,7α-diol was higher and 26-hydroxylation of 5β-cholestane-3α,7α-diol markedly lower in rats with biliary obstruction. Both 25- and 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol were inhibited markedly. Lithium aluminum hydride reduction of methylated extracts of incubations with 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol showed that the decrease in extent of 26-hydroxylation was not due to a conversion of the 26-hydroxy derivatives into C_{27}-acids (cf. ref. 4).

There was no marked difference in effect of substrate concentration on reaction rates between con-

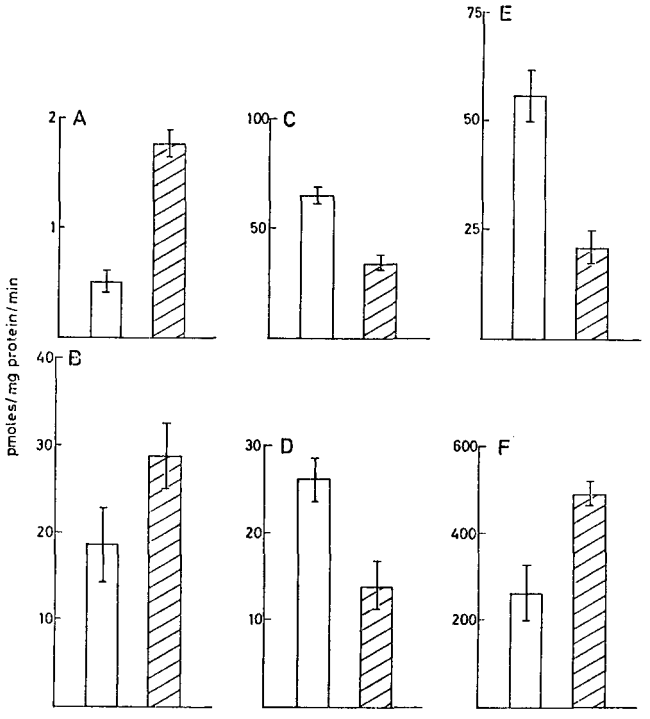

Fig. 3. Effect of biliary obstruction on microsomal 7α-hydroxylation of cholesterol (*A*), 12α-hydroxylation of 5β-cholestane-3α,7α-diol (*B*), 26-hydroxylation of 5β-cholestane-3α,7α-diol (*C*), 25-hydroxylation of cholesterol (*A*). 12α-hydroxylation of 5β-cholestane-3α,7α-diol (*B*), 26-hydroxylation of 5β-cholestane-3α,7α-diol (*C*), 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol (*D*), 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (*E*), and 6β-hydroxylation of lithocholic acid (*F*). The values are the means ± SE of the means of results obtained with four rats. Open bars, control rats; cross hatched bars, obstructed rats.

trol rats and rats with biliary obstruction. In Fig. 2 is shown the effect of substrate concentration on the two microsomal side chain hydroxylations primarily affected by biliary obstruction, 25- and 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol.

Effect of disintegration of mitochondrial membrane structure

Table 1 shows the effect of membrane disintegration by the swelling–condensing–sonication method on mitochondrial 26-hydroxylation. Membrane disintegration of mitochondrial fraction from sham-operated rats resulted in a stimulation of 26-hydroxylation of cholesterol up to the level found in mitochondrial fraction from rats with biliary obstruction. On the other hand, membrane disintegration did not further increase the extent of 26-hydroxylation of cholesterol by mitochondrial fraction from rats with biliary obstruction. When 5β-cholestane-3α,7α 12α-triol was used as substrate, membrane disintegration resulted in a twofold stimulation in the sham-operated rats but had no effect in the rats with biliary obstruction.

DISCUSSION

In a previous report from this laboratory it was shown that biliary obstruction was associated with several effects on hydroxylations in the biosynthesis and metabolism of bile acids (3). In this report the time course of the effects was also followed and it was found that the effects stabilized between 72 and 96 hr. The main effects observed were stimulation of the 7α-hydroxylation of cholesterol and of the 6β-hydroxylation of taurochenodeoxycholic acid and lithocholic acid. Stimulation of 7α-hydroxylation of cholesterol, the rate-limiting step in overall bile acid biosynthesis, is noteworthy since presently available information indicates a decrease in overall bile acid biosynthesis in biliary obstruction (2). No explanation for the effect on 7α-hydroxylation could be offered. The stimulation of 6β-hydroxylation correlated with the increase in β-muricholic acid production associated with biliary obstruction. The present report confirms previous results concerning 7α-hydroxylation of cholesterol and 6β-hydroxylation. In the present experiments 6β-hydroxylation was assayed only with lithocholic acid as substrate. There is strong evidence that the same system catalyzes 6β-hydroxylation of lithocholic acid and of taurochenodeoxycholic acid (3, 10).

It should be pointed out that these reactions were assayed for reasons of comparison. The present results also show that biliary obstruction is associated with effects on side chain hydroxylations of C_{27}-steroids. Mitochondrial 26-hydroxylation of cholesterol and of steroids that are supposed to be early intermediates in bile acid biosynthesis was stimulated, whereas mitochondrial 26-hydroxylation of later intermediates was either unaffected or inhibited (**Fig. 4**). Microsomal 26-hydroxylation of 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol was inhibited.

TABLE 1. Effect of membrane disintegration by the swelling–condensing–sonication method on mitochondrial 26-hydroxylation of cholesterol[a] and 5β-cholestane-3α,7α,12α-triol[b]

Intact Mitochondria				Membrane-Disintegrated Mitochondrial Fraction			
Cholesterol		5β-Cholestane-3α,7α,12α-triol		Cholesterol		5β-Cholestane-3α,7α,12α-triol	
Control	Obstructed	Control	Obstructed	Control	Obstructed	Control	Obstructed
pmol/mg protein/mg							
6.3	16.5	42.0	10.5	17.0	21.3	84.0	14.7

[a] 0.26 μmol.
[b] 0.24 μmol.

It should be pointed out that in all instances hydroxylation of exogenously added substrate was assayed. It can be argued that biliary obstruction could lead to accumulation of cholesterol, of intermediates in the conversion of cholesterol into bile acids, and of bile acids, and that the observed decreases in rates of hydroxylation in rats with biliary obstruction could depend on dilution of added substrates by endogenous substrates. If this argument were valid it could follow that the stimulatory effects observed in the rats with biliary obstruction would be due to lower than normal levels of endogenous substrates. Of the substrates studied in the present investigation, only cholesterol appears to be present in appreciable quantities in mitochondria and microsomes. As mentioned in Methods, there was a moderate increase in cholesterol content of mitochondrial fraction from obstructed rats. In incubations with mitochondrial fraction, the amount of exogenous cholesterol far exceeds that of endogenous cholesterol, making moderate changes in content of endogenous cholesterol of limited importance (cf. ref. 7 and 9). In the microsomal fraction, the amounts of endogenous cholesterol were practically the same in the two groups of rats. Estimations of quantity of intermediates in bile acid biosynthesis present in the liver have been made only for 5-cholestene-3β,7α-diol. By isotope dilution techniques Mitropoulos and Balasubramaniam (11) found about 40 ng of this compound per mg protein in the microsomal fraction of rat liver homogenate. The amount of cholesterol per mg protein is about 25 μg. On the other hand, Björkhem and Danielsson (12), using a gas–liquid chromatographic–mass spectrometric method, were unable to detect any 5-cholestene-3β,7α-diol. Their method would have detected amounts above 10 ng per mg protein.

It might be mentioned that a number of unsuccessful attempts have been made over the years in this laboratory to isolate intermediates from liver. However, it is conceivable that intermediates may accumulate under conditions of biliary obstruction. This possibility has not been analyzed in detail. In the cases where radio–gas chromatography was used in analysis of products formed in incubations, no noticeable differences between control rats and rats with biliary obstruction were seen. Greim et al. (13) have reported that the levels of bile acids in the liver increase up to fourfold after 3 days of biliary obstruction. The increased levels of bile acids could possibly influence the various reactions studied. However, 7α-hydroxylation of cholesterol, which according to previous experience is particularly sensitive to bile acids, was actually stimulated.

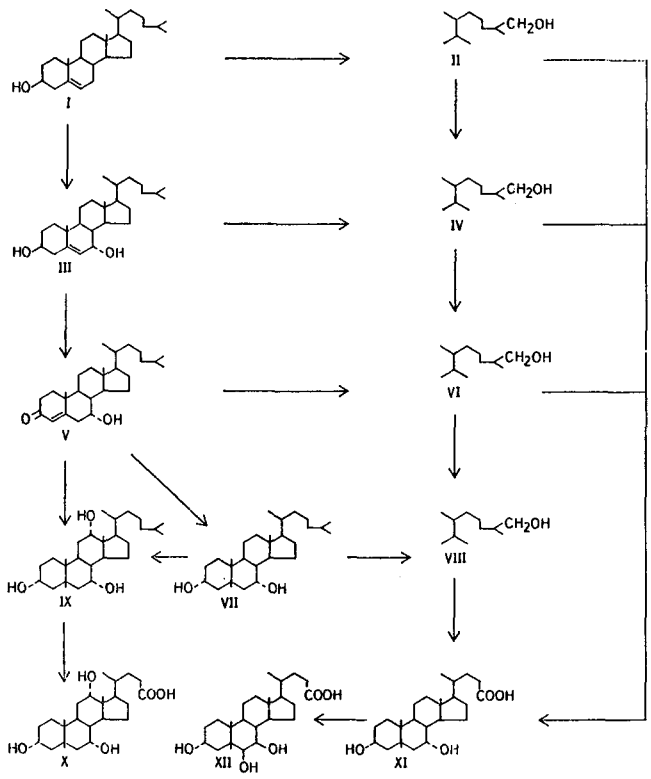

Fig. 4. Some steps in the conversion of cholesterol into bile acids in the rat. I, cholesterol; II, 5-cholestene-3β,26-diol; III, 5-cholestene-3β,7α-diol; IV, 5-cholestene-3α,7β,26-triol; V, 7α-hydroxy-4-cholesten-3-one; VI, 7α,26-dihydroxy-4-cholesten-3-one; VII, 5β-cholestane-3α,7α-diol; VIII, 5β-cholestane-3α,7α,26-triol; IX, 5β-cholestane-3α,7α,12α-triol; X, cholic acid; XI, chenodeoxycholic acid; XII, β-muricholic acid.

The question is also how much of the bile acids accumulated in the liver are present in mitochondria and microsomes, the cell fractions used in the present study. There is no information on the subcellular distribution of bile acids after 4 days of biliary obstruction. Leuschner et al. (14) have examined the distribution after 17 days of obstruction and found more than 70% in the soluble fraction, about 25% in the mitochondrial fraction, and the remainder in the microsomal fraction. In a nonobstructed rat liver there appears to be considerably more in the microsomal fraction and less in the soluble fraction. In the interpretation of the results of the present investigation, the inhibition of microsomal 26-hydroxylation of 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol in biliary obstruction plays a major role. It does not seem likely that this inhibition could be explained by accumulation of bile acids in this subcellular fraction in view of the results reported by Leuschner et al. (14). Also, it should again be pointed out that some microsomal hydroxylations were stimulated. With respect to the mitochondrial hydroxylations, it appears from the

work of Leuschner et al. (14) that no dramatic changes in content of bile acids in this subcellular fraction can be expected upon biliary obstruction.

The differing effects on side chain hydroxylations could explain, at least in part, the increase in formation of chenodeoxycholic acid and its metabolites, α- and β-muricholic acid, and the decrease in formation of cholic acid associated with biliary obstruction. A number of previous studies in which the metabolism of hypothetical intermediates have been examined in rats with a biliary fistula (cf. Fig. 4) indicate that C_{27}-steroids carrying a 26-hydroxyl group but no 12α-hydroxyl group are converted predominantly into chenodeoxycholic acid and its metabolites (15). Examples of such C_{27}-steroids are 5-cholestene-3β,26-diol, 5-cholestene-3β,7α,26-triol, 7α,26-dihydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α,26-triol. Quantitatively, the relative roles of the mitochondrial and the microsomal 26-hydroxylase systems in bile acid biosynthesis have not been established but some data indicate a more important role for the microsomal system under normal conditions (16). Whereas the mitochondrial system catalyzes 26-hydroxylation of many different C_{27}-steroids, the microsomal system shows a marked specificity for 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol. In spite of the broad substrate specificity of the mitochondrial system, several lines of evidence indicate that under normal conditions 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol are also the main substrates for the mitochondrial 26-hydroxylase system (4).

When the results of the present investigation are viewed in light of previous results concerning pathways of bile acid biosynthesis, it appears that the findings in vitro are consistent with the change in pattern of bile acid biosynthesis in biliary obstruction. The increase in synthesis of chenodeoxycholic acid and its metabolites and the decrease in synthesis of cholic acid could well result from the combination of a stimulation of mitochondrial 26-hydroxylation of cholesterol, 5-cholestene-3β,7α,diol, and 7α-hydroxy-4-cholesten-3-one with the inhibition of mitochondrial as well as microsomal 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol. The findings also indicate that mitochondrial 26-hydroxylation assumes a greater role under conditions of biliary obstruction than under normal conditions.

The conclusion that mitochondrial 26-hydroxylation becomes more important in biliary obstruction may be valid also for man. 3β-Hydroxy-5-cholenoic acid, which is present in urine from infants with biliary atresia (17, 18), must be formed through an initial 26-hydroxylation of cholesterol. In man as well as in the rat, this reaction is catalyzed only by the mitochondrial fraction (4, 19).

Present information concerning pathways for bile acid formation in the rat indicates that oxidation of the C_{27}-steroid side chain occurs predominantly through 26-hydroxylation followed by conversion to C_{27}-acids (20). Shefer et al. (21) have reported recently on the occurrence in man of a pathway for cholic acid formation involving 25- and 24-hydroxylation of the side chain. If such a pathway were of importance in the rat under normal conditions, it would be, at any rate, suppressed under conditions of biliary obstruction since the 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol was inhibited markedly.

The present results do not provide any conclusive information concerning the mechanisms of the effects of biliary obstruction on the hydroxylations. Particularly in the case of the mitochondrial hydroxylations, it would appear conceivable that the effects might be due to membrane damage. When membrane-disintegrated mitochondrial preparations were used, 26-hydroxylation of cholesterol was stimulated to the same extent as in preparations from rats with biliary obstruction. On the other hand, 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol was stimulated in membrane-disintegrated preparations but inhibited in preparations from rats with biliary obstruction. These results are difficult to reconcile with membrane damage as a single explanation for the effects on mitochondrial hydroxylations observed in biliary obstruction.

The results of the present investigation may bear on the problem of the mechanisms controlling the proportion of cholic acid and chenodeoxycholic acid synthesized from cholesterol. Previous studies with hyperthyroid rats, in which the normal ratio between the two bile acids is reversed, indicate that the ratio between microsomal 26-hydroxylase activity and microsomal 12α-hydroxylase activity is of importance (16). In hyperthyroid rats, this ratio is increased markedly and in hypothyroid rats it is decreased as compared to euthyroid rats. It is apparent from the present results that an interplay between microsomal 12α- and 26-hydroxylase activities is not an important factor in regulation of cholic acid and chenodeoxycholic acid biosynthesis under conditions of biliary obstruction since the observed effects on these activities should result if anything in a proportional increase in cholic acid biosynthesis. Instead, it appears that in biliary obstruction the ratio between mitochondrial and microsomal 26-hydroxylase activities is important in this respect. Whether or not this rela-

tionship between 26-hydroxylase activities plays a role under normal conditions cannot be assessed at present.

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REFERENCES

1. Mahowald, T. A., J. T. Matschiner, S. L. Hsia, E. A. Doisy, Jr., W. H. Elliott, and E. A. Doisy. 1957. Bile Acids III. Acid I: the principal bile acid in urine of surgically jaundiced rats. *J. Biol. Chem.* **225:** 795–802.
2. Boyd, G. S., M. A. Eastwood, and N. MacLean. 1966. Bile acids in the rat: studies in experimental occlusion of the bile duct. *J. Lipid Res.* **7:** 83–94.
3. Danielsson, H. 1973. Effect of biliary obstruction on formation and metabolism of bile acids in rat. *Steroids.* **22:** 567–579.
4. Björkhem, I., and J. Gustafsson. 1973. ω-Hydroxylation of steroid side-chain in biosynthesis of bile acids. *Eur. J. Biochem.* **26:** 201–212.
5. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. Separation and some enzymatic properties of the inner and outer membranes of rat liver mitochondria. *Methods Enzymol.* **10:** 448–463.
6. Björkhem, I., and H. Danielsson. 1975. 7α-Hydroxylation of exogenous and endogenous cholesterol in rat-liver microsomes. *Eur. J. Biochem.* **53:** 63–70.
7. Gustafsson, J. 1976. On the heterogeneity of the mitochondrial C_{27}-steroid 26-hydroxylase system. *J. Lipid Res.* **17:** 366–372.
8. 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.
9. Björkhem, I., and J. Gustafsson. 1974. Mitochondrial ω-hydroxylation of cholesterol side chain. *J. Biol. Chem.* **249:** 2528–2535.
10. Björkhem, I., and H. Danielsson. 1974. Hydroxylations in biosynthesis and metabolism of bile acids. *Mol. Cell. Biochem.* **4:** 79–95.
11. Mitropoulos, K. A., and S. Balasubramaniam. 1972. Cholesterol 7α-hydroxylase in rat liver microsomal preparations. *Biochem. J.* **128:** 1–9.
12. 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.
13. Greim, H., D. Trülzch, J. Roboz, K. Dressler, P. Czygan, F. Hutterer, F. Schaffner, and H. Popper. 1972. Bile acids in normal rat livers and in those after bile duct ligation. *Gastroenterology.* **63:** 837–845.
14. Leuschner, U., W. Kurtz, A. Alfurayh, and W. Erb. 1974. Die Verteilung von Gallensäuren in der Leberzelle bei extrahepatischer Cholostas. *Z. Gastroenterol.* **12:** 163–168.
15. Danielsson, H. 1963. Present status of research on catabolism and excretion of cholesterol. *Adv. Lipid Res.* **1:** 335–385.
16. Björkhem, I., H. Danielsson, and J. Gustafsson. 1973. On the effect of thyroid hormone on 26-hydroxylation of C_{27}-steroids in rat liver. *FEBS Lett.* **31:** 20–22.
17. Norman, A., and B. Strandvik. 1971. Formation and metabolism of bile acids in extrahepatic biliary atresia. *J. Lab. Clin. Med.* **78:** 181–193.
18. Makino, I., J. Sjövall, A. Norman, and B. Strandvik. 1971. Excretion of 3β-hydroxy-5-cholenoic and 3α-hydroxy-5α-cholanoic acids in urine of infants with biliary atresia. FEBS Lett. **15:** 161–164.
19. Björkhem, I., J. Gustafsson, G. Johansson, and B. Persson. 1975. Biosynthesis of bile acids in man: hydroxylation of the C_{27}-steroid side chain. *J. Clin. Invest.* **55:** 478–486.
20. Danielsson, H. 1973. Mechanisms of bile acid biosynthesis. *In* The Bile Acids, vol. 2. P. P. Nair and D. Kritchevsky, editors. Plenum Press, New York, N.Y. 1–32, 305–306.
21. Shefer, S., F. W. Cheng, B. Dayal, S. Hauser, G. S. Tint, G. Salen, and E. H. Mosbach. 1976. A 25-hydroxylation pathway of cholic acid biosynthesis in man and rat. *J. Clin. Invest.* **57:** 897–903.