An in vivo evaluation of the quantitative significance of several potential pathways to cholic and chenodeoxycholic acids from cholesterol in man

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Abstract The present study was designed to obtain more definitive information in man on the metabolic pathways to chenodeoxycholic acid and to cholic acid via a pathway not involving an initial 7α-hydroxylation of cholesterol. Four bile fistula patients were administered consecutively two or more of the following ^{3}H-labeled bile acid intermediates: 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, 5β-cholestane 3α,7α,26-triol, 26-hydroxycholesterol, 7α,26-dihydroxy-4-cholesten-3-one, and 5-cholestene-3β,-12α-diol. Both 7α-hydroxy[7β-^{3}H]cholesterol and 7α-hydroxy-4-[6β-^{3}H]cholesten-3-one were efficiently converted to bile acids and preferred chenodeoxycholic acid over cholic acid. The specific activity time curves indicated that a portion of cholic acid synthesis did not pass through 7α-hydroxycholesterol. [^{3}H]26-Hydroxycholesterol and [^{3}H]-5-cholestene 3β,12α-diol, two potential intermediates of this bypass pathway to cholic acid, were poorly converted to primary bile acids (10 to 27%). The [^{3}H]26-hydroxycholesterol preferred chenodeoxycholic over cholic acid by about 4 to 1. The [^{3}H]5-cholestene 3β,12α-diol formed cholic acid in low yield (10 to 20%). It is concluded that pathways to primary acids from cholesterol through 26-hydroxycholesterol and 5-cholestene 3β,12α-diol are probably of minor quantitative significance. A selective pathway to chenodeoxycholic acid via 26-hydroxylation of 7α-hydroxy-4-cholesten-3-one was also investigated. The 5β-cholestane 3α,7α,26-triol was converted in about equal amounts to cholic and chenodeoxycholic acids. The 7α-hydroxy-4-cholesten-3-one was also efficiently converted to both bile acids but preferred chenodeoxycholic acid. The most efficient precursor of chenodeoxycholic acid was 7α,26-dihydroxy-4-cholesten-3-one, which was efficiently converted to primary bile acids; chenodeoxycholic acid was preferred over cholic acid by approximately 7 to 1. These findings suggest the presence of a major pathway to chenodeoxycholic acid via the 26-hydroxylation of 7α-hydroxy-4-cholesten-3-one and intermediate formation of 7α,26-dihydroxy-4-cholesten-3-one.—**Swell L., J. Gustafsson, C. C. Schwartz, L. G. Halloran, H. Danielsson, and Z. R. Vlahcevic.** An in vivo evaluation of the quantitative significance of several potential pathways to cholic and chenodeoxycholic acids from cholesterol in man. *J. Lipid Res.* 1980. **21:** 455–466.

Supplementary key words bile acids · metabolic pathways · 7α-hydroxylation · multiple pathways

Cholic and chenodeoxycholic acids represent the major catabolic end products of cholesterol metabolism in man. These bile acids are synthesized from cholesterol in the liver cell by a series of reactions which involve epimerization of the 3β-hydroxy group, reduction of the Δ^5 double bond to produce the 5β-configuration, introduction of α hydroxy groups at C_7 or C_7 and C_{12} positions and oxidative degradation of the side chain by 3 carbons. Present concepts of the nature and sequence of enzymatic reactions involved in these transformations have been derived to a large extent from studies in the rat (1). The initial steps in the pathway are thought to be common for both primary bile acids and entail the microsomal 7α-hydroxylation of cholesterol to form 7α-hydroxycholesterol which is then converted to 7α-hydroxy-4-cholesten-3-one. The latter compound is considered to be the last intermediate common to the synthesis of cholic acid and chenodeoxycholic acid. This unsaturated ketone can then be reduced by soluble enzymes to 5β-cholestane 3α,7α-diol or 12α-hydroxylated in microsomes to form 7α,12α-dihydroxy-4-cholesten-3-one. This latter compound is then reduced to 5β-cholestane 3α,7α,12α-triol. According

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

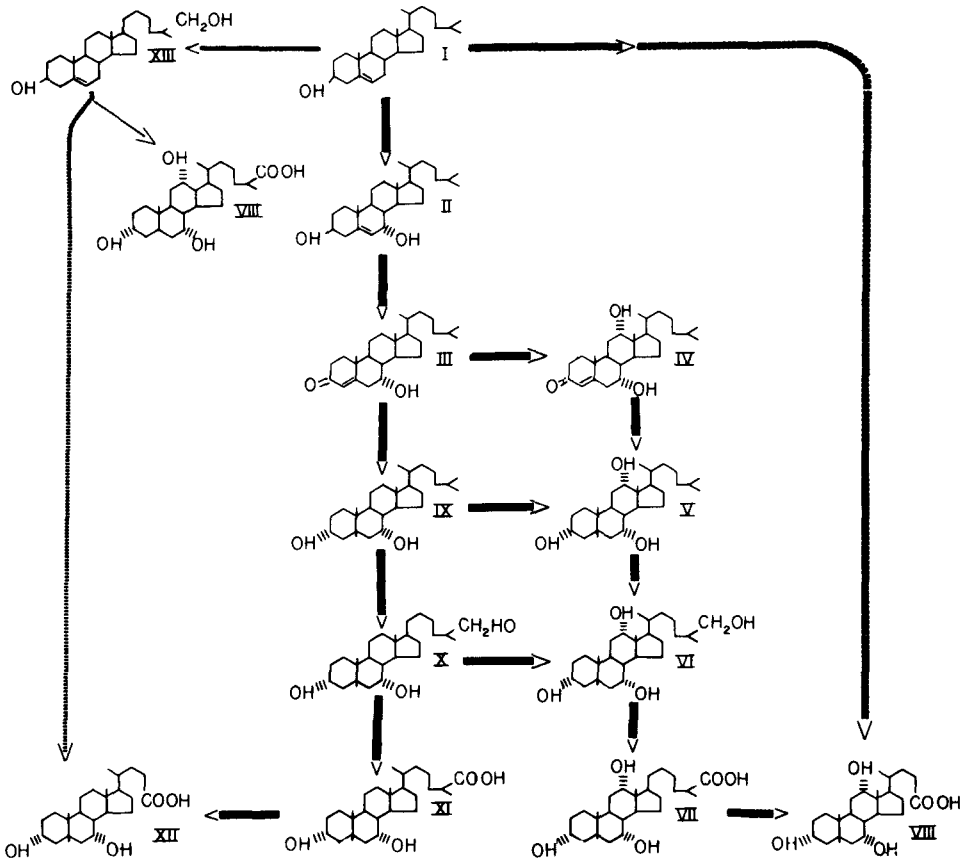

Fig. 1. Schematic representation of proposed multiple pathway to cholic and chenodeoxycholic acid in man. I, Cholesterol; II, 7α-hydroxycholesterol; III, 7α-hydroxy-4-cholesten-3-one; IV, 7α,12α-dihydroxy-4-cholesten-3-one; V, 5β-cholestane 3α,7α,12α-triol; VI, 5β-cholestane 3α,7α,12α,26-tetrol; VII, 3α,7α,-12α-trihydroxy-5β-cholestanoic acid; VIII, cholic acid; IX, 5β-cholestane 3α,7α-diol; X, 5β-cholestane 3α,7α,26-triol; XI, 3α,7α-dihydroxy-5β-cholestanoic acid; XII, chenodeoxycholic acid; XIII, 26-hydroxy-cholesterol.

to the present view, side chain oxidation is initiated by microsomal or mitochondrial 26-hydroxylation of 5β-cholestane 3α,7α-diol and 5β-cholestane 3α,7α,-12α-triol and the resultant products are oxidized to chenodeoxycholic and cholic acids, respectively.

Although there has been limited data available in man on the nature of the biosynthetic pathways to bile acids, it has been shown that there are a number of metabolic steps common to the pathway observed in the rat (2–8). Recent in vivo studies in man (9–12) have demonstrated that cholesterol can be degraded to primary bile acids via a number of routes which may have important quantitative significance (11). These recent observations are schematically shown in **Fig. 1.** The pertinent points in relation to these newer findings are: *a*) a significant fraction (25 to 30%) of the total cholic acid may be synthesized in subjects with biliary diversion by an unknown pathway directly from cholesterol which bypasses 7α-hydroxy-cholesterol; *b*) the remainder of cholic acid which is formed via the conventional 7α-hydroxycholesterol pathway can arise by 12α-hydroxylation of several intermediates, i.e., 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one and 5β-cholestane 3α,7α,-26-triol; and *c*) side chain oxidation does not prevent additional changes on the nucleus since administered 5β-cholestane 3α,7α,26-triol can be 12α-hydroxylated and converted to cholic acid in good yield. These findings also suggest that 7α-hydroxy-4-cholesten-3-one may not be the only branching point for primary bile acid synthesis in man.

In the earlier studies (10) the existence of a selective pathway to chenodeoxycholic acid was postulated since 7α-hydroxy-4-cholesten-3-one was converted to chenodeoxycholic acid to a greater extent than 5β-cholestane 3α,7α-diol. These findings strongly suggested the presence of an alternative route to chenodeoxycholic acid which could entail the 26-hydroxylation of 7α-hydroxy-4-cholesten-3-one to form 7α,26-dihydroxy-4-cholesten-3-one.

The present investigation was designed to provide additional information on the nature of these recently

demonstrated metabolic pathways to cholic and chenodeoxycholic acid in man. Patients with bile fistula were administered consecutively two or more potential intermediates of the bile acid pathways; the efficiency of the conversion to primary bile acid was determined and evaluated on a comparative basis. The findings of the present report strongly support the existence in man of a major pathway to chenodeoxycholic acid which proceeds via the 26-hydroxylation of 7α-hydroxy-4-cholesten-3-one. The compound 7α,26-dihydroxy-4-cholesten-3-one was found to be the most efficient precursor of chenodeoxycholic acid tested in this or in the previous studies (9–12). Pathways to primary bile acids from cholesterol through 26-hydroxycholesterol and 5-cholestene-3β,12α-diol were found to be of minor quantitative significance.

EXPERIMENTAL PROCEDURE

Labeled and reference compounds

The [4-^{14}C]cholesterol was obtained from New England Nuclear, Boston, MA and checked for purity by silicic column chromatography, thin-layer chromatography and digitonin precipitation. If less than 97% pure, it was purified by silicic column chromatography and/or thin-layer chromatography.

The 7α-hydroxy-4-[6β-^{3}H]cholesten-3-one was prepared (13) by reduction of 6α,7α-epoxycholest-4-en-3-one with lithium aluminum [^{3}H]hydride followed by selective oxidation of the C-3 hydroxyl group with manganese dioxide. The material was purified by thin-layer chromatography with toluene–ethyl acetate 1:1 (v/v) as the solvent system. Specific activity of the product was 130 μCi/μmol.

The 5β-[3β-^{3}H,7β-^{3}H]cholestane 3α,7α,26-triol was prepared (14) from the methyl ester of 3α,7α-dihydroxy-5β-[3β-^{3}H,7β-^{3}H]cholestanoic acid by reduction with lithium aluminum hydride. The product was subjected to thin-layer chromatography (15) to determine the ratio of *R* to *S* isomers; the major isomer was of the *S* type which accounted for 80% of the total product. The small amount (20%) of the *R*-isomer present was probably formed during the hydrolysis of the C_{27} acid. The final product was purified by thin layer chromatography with ethyl acetate as the solvent. Specific activity of the product was 3.7 μCi/μmol.

The 7α-hydroxy-[7β-^{3}H]cholesterol was prepared by reduction of 7-keto-cholesterol with ^{3}H-labeled sodium borohydride (16). The 7α- and 7β-isomers were separated by thin-layer chromatography with diethyl ether as the solvent system. Specific activity of the product was 1 mCi/μmol.

The 5-[G-^{3}H]cholesten-3β,12α-diol (12α-hydroxycholesterol) was prepared as described previously (17) and then exposed to tritium exchange labeling by the Wilzbach procedure. Specific activity of the product was 2 μCi/μmol.

The 7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one was biosynthesized from 7α-hydroxy-4-[6β-^{3}H]cholesten-3-one in incubations with rat liver mitochondria and isocitrate (18). The mitochondrial fraction was prepared according to Sottocasa et al. (19) and was contaminated by less than 2% of microsomal protein as judged by assay of the microsomal 12α-hydroxylase activity. The product was purified by thin-layer chromatography and the identity confirmed as described earlier (6, 18). Radio-gas–liquid chromatography of the product showed that >98% consisted of 7α,26-dihydroxycholest-4-en-3-one. According to earlier work by Berseus (20) and to recent work by Popjak et al. (21), the mitochondrial fraction hydroxylates the 26 pro-*S* methyl-group. The compound used in the present investigation thus should have the 25-*S* configuration. Specific radioactivity of the product was 130 μCi/μmol.

The 5-[G-^{3}H]cholestene-3β,26-diol (26-hydroxycholesterol) was prepared from the methyl ester of generally tritium labeled 3β-hydroxy-5-cholestenoic acid by reduction with lithium aluminum hydride. The [G-^{3}H]3β-hydroxy-5-cholestenoic acid was prepared from 3β-hydroxy-5-cholestenoic acid by electrolysis in a similar manner to the earlier described preparation of 3β,7α-dihydroxy-5-cholestenoic acid (22). The material consisted of equal parts of the 25-*S* and 25-*R*-isomers. Specific radioactivity of the product was 400 μCi/μmol.

All of the administered labeled components were purified by thin-layer chromatography immediately prior to administration to the patients.

Patients

Four post-cholecystectomy female patients with a T-tube in the common bile duct were studied; informed consent was obtained. The study was approved by the Committee on the Conduct of Human Research, Medical College of Virginia. The experiments were carried out 10–14 days after surgery. The patients were in good nutrition and received a regular hospital diet, and their liver function tests (serum albumin, SGOT, alkaline phosphatase, and bilirubin) were all normal. Bile was allowed to drain from the T-tube by gravity for 4 to 8 days prior to the start of the experiments to achieve a maximal and constant rate of bile acid synthesis. At the time of the experiments, secondary bile acids (deoxycholic and litho-

cholic) were absent from the bile and stools were acholic. Both of these indicators strongly suggest essentially complete interruption of the enterohepatic circuit.

A known amount of the radioactive labeled compound was dissolved in 0.1 ml of ethanol and slowly added with agitation to 30 ml of 25% sterile human albumin. This solution was then injected, at 9–10 AM, intravenously over a 2-min period. The syringe was rinsed with an additional 10 ml of albumin which was then injected into the patient. The labeled intermediates were administered on consecutive days and the compounds given to each patient are shown in **Table 1.** Patient KI also received simultaneously [4-^{14}C]cholesterol with 7α-hydroxy[7β-^{3}H]cholesterol on the third day of the study.

Bile samples were collected at 20-min intervals for the first 3–4 hr, and then at 1- to 2-hr intervals for the remainder of the study period. The collection period varied from 11.7 to 24 hr, but was usually close to 24 hr. (Table 1). Bile flow was relatively constant during the study period and ranged from 0.5 to 1 ml/min. Bile acid secretion rates for each patient (Table 1) remained relatively constant (±10%) throughout the course of the experiments; there was no evidence for diurnal variation. The bile was monitored for ^{3}H-activity prior to the administration of the next labeled compound and was found to be very low in radioactivity (<twice background).

METHODS

Bile samples were extraced with 20 vol of chloroform–methanol 2:1, filtered and the resulting solution was washed with 1/5 vol of water (23). The chloroform phase was found to contain less than 5% of the administered ^{3}H-activity. The water–methanol phase contained cholic acid, chenodeoxycholic, and other acidic products. Both mass and radioactivity of the bile acids were determined as described earlier (24). Aliquots of the water–methanol phase were autoclaved for 3 hr with 10% KOH; the solution was then adjusted to a pH of 1–2 with HCl. The free bile acids were extracted three times with 50 ml of diethylether and treated with diazomethane to form the methyl esters. An aliquot of the methyl ester fraction was subjected to GLC to quantitate the individual bile acids; 5α-cholestane was used as an internal standard. The stationary phase was 3% HI EFF 8BP (Applied Science Laboratories, State College, PA). Another aliquot of the methyl ester fraction was subjected to TLC on silica gel G with a solvent system of ethyl acetate–isooctane–acetic acid 5:5:1 (v/v/v); the plates were developed twice. The methyl cholate and methyl chenodeoxycholate zones were scraped from the plate, and eluted with ethanol–chloroform–water–acetic acid 100:32:20:2 (v/v/v/v). The mass of the primary bile acids was determined by GLC and radioactivity by liquid scintillation counting; quench correction was applied by the external standard method (Mark III, Tracor Analytic, Silver Spring, MD). Radioactivity was also determined on the original bile and all subsequent extracts. From 5 to 40% of the radioactivity on the TLC plates was found in zones other than cholic acid and chenodeoxycholic acid (**Table 2**). No attempt was made to further characterize these acidic products. The identity of the labeled cholic and chenodeoxycholic acids isolated from the bile was evaluated by migration on thin-layer plates and the homogeneity of mass and label, as judged by no significant change in specific activity upon repeated crystallization of the methyl esters, and also by their elution pattern on silicic acid columns (9).

Interpretation of data

The in vivo data obtained in the bile fistula patients were subject to the following assumptions and interpretations. The bile fistula patient provided an important basis for evaluating the quantitative conversion of the administered labeled compounds to primary bile acids. These patients do not have an intervening bile acid pool and therefore all of the bile formed by the liver can be collected externally via the T-tube. Thus all of the newly synthesized bile acids as secreted from the hepatic precursor site can be quantitated. Also, since these patients were found to very rapidly metabolize the administered labeled compounds, it was possible to give each patient a series of labeled precursors on consecutive days. This approach has the important attribute of minimizing patient to patient variation and allows for the evaluation of multiple precursors to cholic and chenodeoxycholic acids in the same patient.

Inherent to the interpretation of the data from the present and previous in vivo studies (3–5, 7, 9), is the assumption that the intravenously administered, presumed intermediates are rapidly and exclusively removed by the liver and that they are homogenously mixed with similar endogenous substrates in the appropriate organelle site. With the presently available technology however, this assumption cannot be objectively validated. This general approach has been utilized in all previous in vivo studies in man, and has in general given results which are compatible with in vitro findings (1, 2, 6) on the metabolism of bile acid precursors by liver cell fractions.

TABLE 1. Labeled compounds administered to patients and bile acid secretion rates

Patient[a]	Labeled Compound Administered	Radioactive Dose	Collection Period[b]	Bile Acid Secretion Rates[c]	
				Cholic	Chenodeoxycholic
		μCi	*hr*	*μmol/min*	
HU	26-hydroxy-[G-^{3}H]cholesterol	0.97	23.0	3.59	1.46
	5-[G-^{3}H]cholesten-3β,12α-diol	0.82	20.3	3.43	1.41
	7α-hydroxy-[7β-^{3}H]cholesterol	1.06	19.9	3.55	1.49
	7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one	0.77	22.2	3.30	1.30
KI	7α-hydroxy-4-[6β-^{3}H]cholesten-3-one	1.54	19.4	4.36	1.67
	7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one	0.60	22.1	4.60	1.87
	7α-hydroxy-[7β-^{3}H]cholesterol	1.07	23.3	5.00	2.00
	[4-^{14}C]cholesterol	10.00			
OV	7α-hydroxy-4-[6β-^{3}H]cholesten-3-one	1.05	11.7	1.47	0.95
	7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one	0.73	11.7	0.97	0.60
	5β-[3β-^{3}H,7β-^{3}H]cholestane 3α,7α,26-triol	0.61	13.0	1.04	0.59
WI	26-hydroxy-[G-^{3}H]cholesterol	0.87	23.8	3.03	1.26
	5-[G-^{3}H]cholesten-3β,12α-diol	0.45	24.0	3.31	1.40

[a] The patients were all females ages 43, 47, 48, and 32 years respectively. The labeled intermediates were administered intravenously on consecutive days. Patient KI received [^{3}H]7α-hydroxycholesterol and [^{14}C]cholesterol simultaneously on the final day of the study.

[b] Represents the total time period bile was collected following administration of the labeled compounds.

[c] Represent the average bile acid secretion rates during the period of the study.

The criteria for evaluating the quantitative significance of a given compound on a pathway to cholic and chenodeoxycholic acid were the rapidity and efficiency of conversion of the precursor to bile acids and the specific activity relationships between the two primary bile acids. Specific activity curves for cholic and chenodeoxycholic and primary bile acid synthesis rates (secretion) were obtained throughout the period of excretion of the labeled products. It was therefore possible to obtain direct quantitative data on the efficiency and fractional conversion of the labeled compounds to cholic acid and chenodeoxycholic acid and to correlate these data with the proportion of each primary bile acid synthesized endogenously by the liver. If the specific activities of cholic and chenodeoxycholic acids were similar at all times following

TABLE 2. Conversion of administered labeled intermediates to primary bile acids

Patient	Labeled Compound Administered	% Administered ^{3}H-Activity Recovered in Bile			Cholic/Chenodeoxycholic Acid		
		Cholic	Chenodeoxycholic	Total[a]	^{3}H	Synthesis	^{3}H/Synthesis
HU	26-hydroxy-[G-^{3}H]cholesterol	9.1	18.4	59.5	0.40	2.46	0.20
	5-[G-^{3}H]cholesten-3β,12α-diol	12.3	0.0	62.8[b]		2.43	
	7α-hydroxy-[7β-^{3}H]cholesterol	46.3	45.6	104.6	1.02	2.32	0.43
	7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one	18.1	73.6	91.7	0.25	2.38	0.11
KI	7α-hydroxy-4-[6β-^{3}H]cholesten-3-one	41.1	30.1	77.7	1.37	2.61	0.32
	7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one	24.9	69.7	95.0	0.36	2.46	0.15
	7α-hydroxy-[7β-^{3}H]cholesterol	45.4	34.8	80.0	1.30	2.50	0.32
	[4-^{14}C]cholesterol						
OV	7α-hydroxy-4-[6β-^{3}H]cholesten-3-one	31.6	32.7	87.0	0.97	1.55	0.63
	7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one	15.5	45.3	79.8	0.34	1.62	0.21
	5β-[3β-^{3}H,7β-^{3}H]cholestane 3α,7α,26-triol	23.3	22.9	70.2	1.02	1.76	0.58
WI	26-hydroxy-[G-^{3}H]cholesterol	9.2	12.0	56.0	0.77	2.40	0.32
	5-[G-^{3}H]cholesten-3β,12α-diol	10.8	0.0	54.8[b]		2.36	

[a] Represents the total amount of administered ^{3}H-activity recovered in the bile during the period the bile was collected. The difference between the sum of the primary bile acids and the total represents other ^{3}H-labeled products which were principally of an acidic nature, but not identified.

[b] The principal ^{3}H-labeled bile acid found in the bile of patients WI and HU after the administration of 5-[G-^{3}H]cholestene-3β,12α-diol was judged to be deoxycholic acid on the basis of TLC migration.

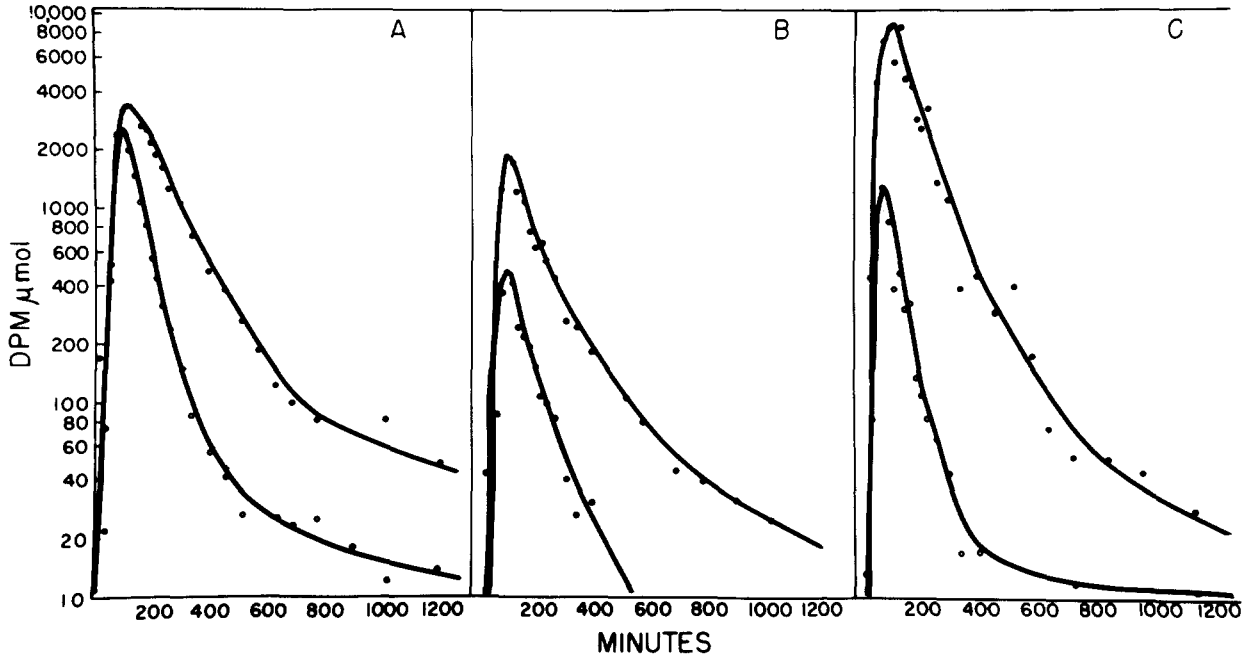

Fig. 2. Specific activity time course curves for cholic [○] and chenodeoxycholic [●] acids following the administration of 7α-hydroxy-[7β-^{3}H]cholesterol (panel A), 26-hydroxy-[G-^{3}H]cholesterol (panel B), and 7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one (panel C) to patient HU.

the administration of an intermediate prior to commitment to either primary bile acid, it is assumed that the synthesis of both bile acids passed through the administered compound. Whether the entire production of both primary bile acids passed through a common intermediate was also dependent on the efficiency of conversion of the compound to primary bile acids. If the specific activities of cholic and chenodeoxycholic acids secreted by the liver were unequal, it is assumed that a portion of one of the primary bile acids was derived in part from a precursor source which did not pass through the administered compound. This implies that the bile acid with the lower specific activity would have been diluted by unlabeled bile acid from a source preceding the administered intermediate. The extent of conversion of a labeled intermediate to either primary bile acid was evaluated by a combination of the efficiency of conversion and a comparison of the cholic to chenodeoxycholic acid ratios by label and by synthesis. The ratios were derived from cholic acid to chenodeoxycholic acid synthesis (or secretion rates) and the fractional conversions of the labeled intermediates to the primary bile acids.

RESULTS

Patient HU

Patient HU received four different intermediates on consecutive days (Table 1). The specific activity time course curves for cholic and chenodeoxycholic acids following the administration of 26-hydroxy-[G-^{3}H]cholesterol, 7α-hydroxy[7β-^{3}H]cholesterol, and 7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one, are shown in **Fig. 2A, B,** and **C.** Following the administration of the labeled bile acid precursors, there was a very rapid rate of incorporation of the ^{3}H-label into primary bile acids. The peak specific activities of the bile acids were reached in 30–60 min and then declined rapidly. Chenodeoxycholic acid had a higher specific activity than cholic acid throughout the course of the experiments. This difference between cholic and chenodeoxycholic acids was particularly pronounced, at the peak, for the 7α,26-dihydroxy-4-cholesten-3-one and 26-hydroxycholesterol compounds. The quantitative recovery data are shown in Table 2. The 26-hydroxy-[G-^{3}H]cholesterol was poorly converted to bile acids; only 27.5% of the administered ^{3}H-activity was found in cholic and chenodeoxycholic acids. The partitioning of recovered ^{3}H-label was in favor of chenodeoxycholic acid (18.4%) over cholic acid (9.1%). Comparison of the ratios of the two primary bile acids by ^{3}H-activity versus endogenous synthesis clearly shows that ^{3}H-labeled 26-hydroxycholesterol was converted to chenodeoxycholic acid over cholic acid by a wide margin (5 to 1). The remaining recovered ^{3}H-activity which was present in the acidic fraction of the bile was substantial (32.1%) but was not further characterized. The labeled 5-cholestene-3β,12α-diol compound was also converted very poorly to primary bile

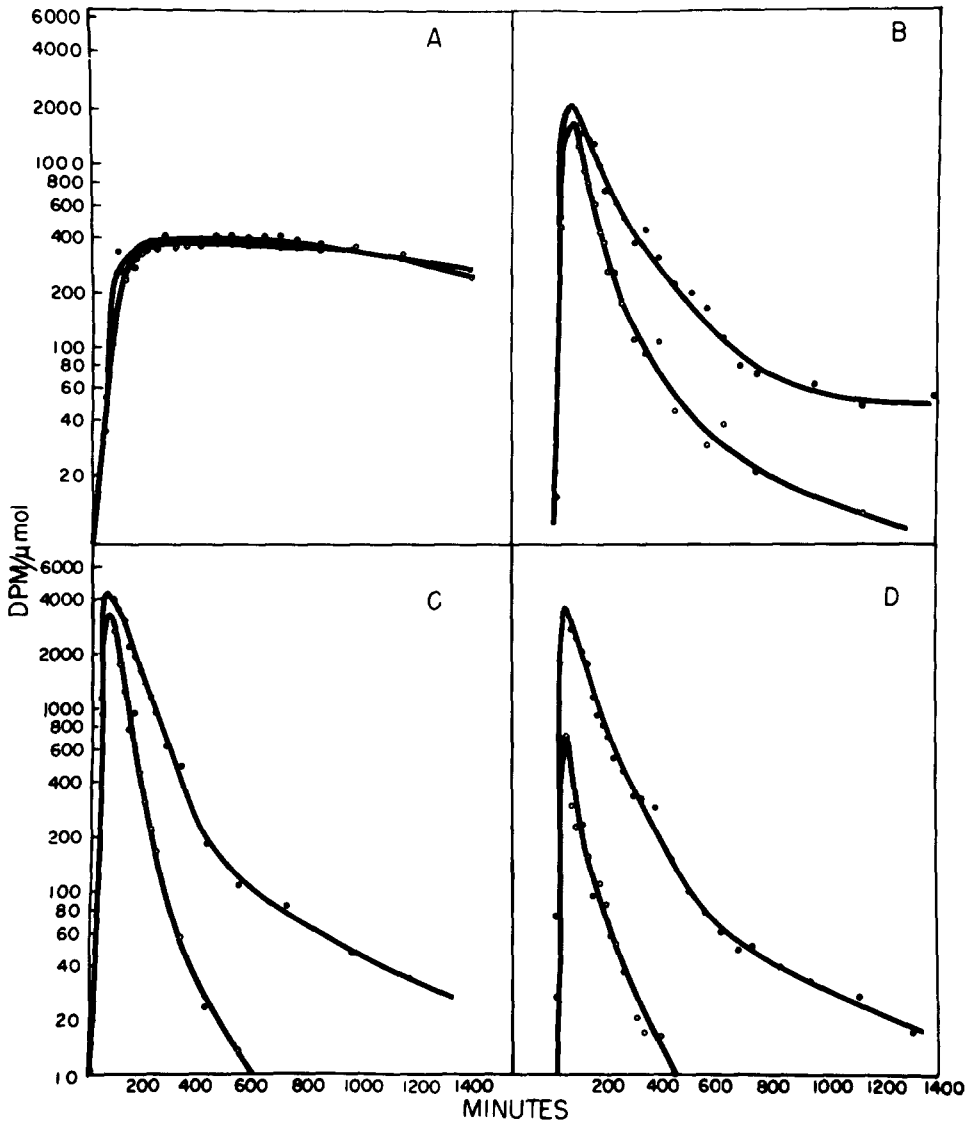

Fig. 3. Specific activity time course curves for cholic [○] and chenodeoxycholic [●] acid following the administration of [4-^{14}C]cholesterol (panel A), 7α-hydroxy-[7β-^{3}H]cholesterol (panel B), 7α-hydroxy-4-[6β-^{3}H]cholesten-3-one (panel C), and 7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one (panel D) to patient KI.

acids. Only 12.3% of the administered ^{3}H-activity was recovered in cholic acid and, as expected, no incorporation into chenodeoxycholic acid was noted. The bulk (40%) of the ^{3}H-activity recovered in the bile was found in the deoxycholic acid zone on TLC. Very efficient conversion of 7α-hydroxy[7β-^{3}H]-cholesterol to primary bile acids occurred. Approximately equal proportions of the administered radioactivity were found in cholic (46.3%) and chenodeoxycholic (45.6%) acids. A comparison of the ratio of cholic to chenodeoxycholic acid by ^{3}H-activity and synthesis indicated that labeled 7α-hydroxycholesterol favored chenodeoxycholic acid over cholic acid by about 2.5 to 1. The 7α,26-dihydroxy-4-cholesten-3-one compound was very efficiently (91.7%) converted to primary bile acids. However, the proportioning of ^{3}H-activity between the bile acids was strikingly in favor of chenodeoxycholic acid. The cholic to chenodeoxycholic ratios by ^{3}H-activity and synthesis indicate that chenodeoxycholic acid was favored over cholic acid by about 9 to 1.

Patient KI

The specific activity time course curves following the administration of the labeled bile acid precursors to patient KI are shown in **Fig. 3A, B, C,** and **D.** The specific activity relationships for cholic and chenodeoxycholic acid followed a similar pattern for this patient as was observed in patient HU. After the administration of 7α-hydroxy-[7β-^{3}H]cholesterol, 7α-

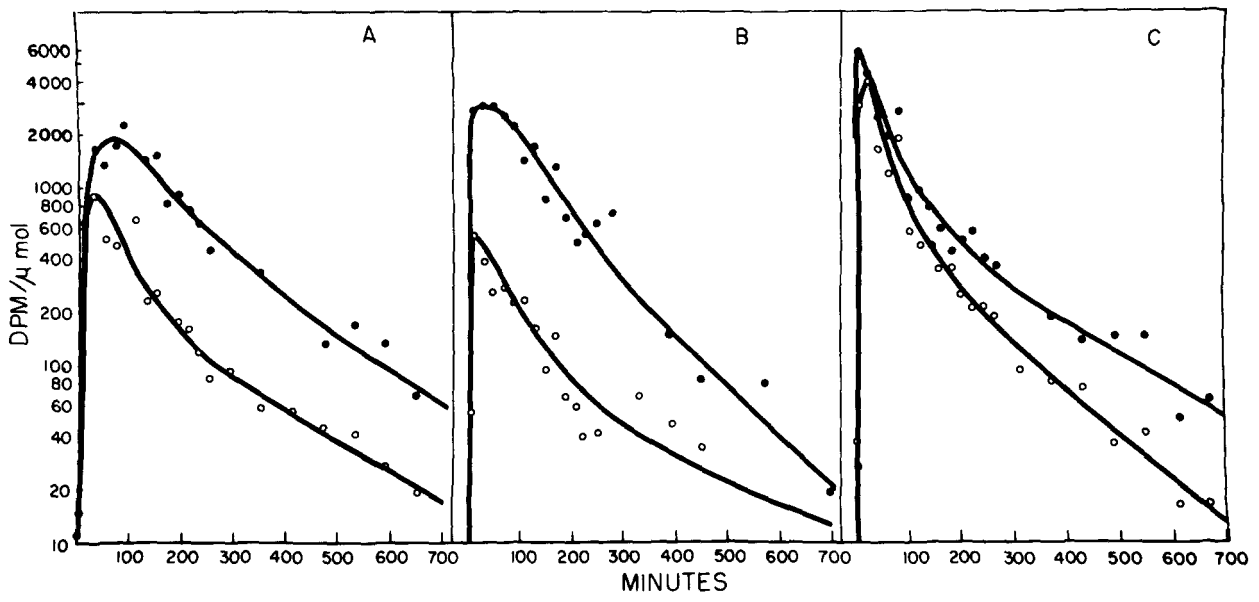

Fig. 4. Specific activity time course curves for cholic [○] and chenodeoxycholic [●] acid following the administration of 7α-hydroxy-4-[6β-^{3}H]cholesten-3-one (panel A), 7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one (panel B), and 5β-[3β-^{3}H,7β-^{3}H]cholestane 3α,7α,26-triol (panel C), to patient OV.

hydroxy-4-[6β-^{3}H]cholesten-3-one, and 7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one, chenodeoxycholic acid had a higher specific activity than cholic acid. This patient also received [^{14}C]cholesterol simultaneously with labeled 7α-hydroxycholesterol. The specific activities of cholic and chenodeoxycholic acids were essentially identical over the 23-hr observation period following the administration of the [^{14}C]cholesterol; by contrast after the administration of 7α-hydroxy-[7β-^{3}H]cholesterol, the specific activity of chenodeoxycholic acid was much higher than cholic acid.

The recovery of administered label in bile acids is shown in Table 2. Excellent conversion to primary bile acids after labeled 7α-hydroxy-4-cholesten-3-one (71.2%), 7α-hydroxycholesterol (80.2%), and 7α,26-dihydroxy-4-cholesten-3-one (94.6%) were noted. Both 7α-hydroxy-4-[6β-^{3}H]cholesten-3-one and 7α-hydroxy[7β-^{3}H]cholesterol showed a preference for chenodeoxycholic acid over cholic acid (from 2 to 3/1) as indicated by comparing the ratios of cholic to chenodeoxycholic acids by ^{3}H-label and endogenous synthesis. The data obtained with the 7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one precursor were particularly striking and show again a more marked preference for chenodeoxycholic acid over cholic acid (6.7 to 1). The latter observations are similar to that observed for patient HU.

Patient OV

The specific activity time course curves are shown in **Fig. 4A, B,** and **C.** The specific activities of cholic and chenodeoxycholic acids following the administration of 7α-hydroxy-4-[6β-^{3}H]cholesten-3-one and 7α,26-dihydroxy-4-[6β-^{3}H]cholesten-3-one were similar to patient HU. The peak specific activities were reached very rapidly and chenodeoxycholic acid had a higher specific activity than cholic acid. In the case of the 5β-[3β-^{3}H, 7β-^{3}H]cholestane 3α,7α,26-triol, the specific activity of chenodeoxycholic was also higher than cholic acid, but the differences were not as great as was observed for 7α-hydroxy-4-cholesten-3-one and 7α,26-dihydroxy-4-cholesten-3-one. The recovery of administered ^{3}H-activity in bile ranged from 70.2% to 81.0%, but the degree of conversion (46.2 to 64.3%) to primary bile acids, although good, was somewhat lower than observed for patients HU and KI. The 7α-hydroxy-4-cholesten-3-one and 5β-cholestane 3α,7α,26-triol favored chenodeoxycholic acid over cholic acid by approximately 1.7 to 1. As with the previous patients HU and KI, the 7α,26-dihydroxy-4-cholesten-3-one compound showed a marked preference for chenodeoxycholic acid over cholic acid (5 to 1). It is noteworthy, as observed in the previous reports (9), that considerable amounts of cholic acid were formed from the 5β-cholestane 3α,7α,26-triol.

Patient WI

This patient received two ^{3}H-labeled intermediates on consecutive days. The specific activity curves (**Fig. 5**) for 26[G-^{3}H]hydroxycholesterol were similar to those observed for patient HU. Table 2 shows that the labeled 26-hydroxycholesterol was poorly (21.2%) converted to bile acids; chenodeoxycholic acid

(12.0%) was favored over cholic acid (9.2%). The 5-cholestene 3β,12α-diol compound was also poorly converted to primary bile acids. No ^{3}H-activity was found in chenodeoxycholic acid and only 10.8% of the administered ^{3}H dose was in cholic acid. The bulk of the radioactivity present in the bile was confined to a large extent (40%) to the deoxycholic acid zone on thin-layer chromatography.

DISCUSSION

Several recent reports (7, 9, 10–12, 25) have provided important new information on the nature of the biosynthetic pathways to primary bile acids in man. The in vivo studies have shown that the degradation of cholesterol to bile acids may involve alternative pathways which have not been readily apparent from the numerous studies carried out in the rat. Several important features distinguish the bile acid pathway in man from what has previously been reported in the rat. There appear to be multiple routes to cholic and chenodeoxycholic acids from cholesterol (11). Compatible with this view are the consistent findings from patient to patient that bile acid precursor substrates, which have not been committed to cholic acid, via 12α-hydroxylation and 26-carboxylation are converted to both cholic acid and chenodeoxycholic acid. This implies that the 7α, 12α, and 26-hydroxylation steps in the pathway are not limited to the substrates indicated in Fig. 1, and also does not support the contention (1) that the bifurcation point to cholic acid and chenodeoxycholic acid exists primarily at the level of 7α-hydroxy-4-cholesten-3-one. It has also been generally accepted that the initial reaction in bile acid synthesis involves the 7α-hydroxylation of cholesterol to form 7α-hydroxycholesterol. This step has also been regarded as the key reaction regulating bile acid synthesis. While the present and previous studies do not contradict the importance of this reaction in the control of bile acid synthesis, they do suggest that the 7α-hydroxylase enzyme may also have other substrates than cholesterol. Specifically, in the previous reports it was shown (11) that a significant portion of cholic acid synthesis appears to arise via a route which bypasses the initial 7α-hydroxylation of cholesterol. The present and previous studies (9, 10) also clearly dispel the generally accepted view based on studies in the rat that 12α-hydroxylation of the steroid ring must precede oxidation at C_{26}. In every patient studied the intermediate 5β-cholestane 3α,7α,26-triol was found to be efficiently converted to both cholic acid and chenodeoxycholic acid.

Studies by Hanson et al. (7) indicated that normal subjects with intact enterohepatic circulation may have a bypass pathway to cholic acid at the level of 7α-hydroxycholesterol since the administration of labeled 7α-hydroxy-4-cholesten-3-one resulted in the formation of cholic acid with a lower specific activity than chenodeoxycholic acid. However, we have demonstrated that following the administration of ^{3}H-labeled 7α-hydroxycholesterol the specific activity of chenodeoxycholic acid also exceeded that of cholic acid. This indicates that unlabeled cholic acid must have been derived from a substrate proximal to 7α-hydroxycholesterol i.e., cholesterol. It was estimated that about 30% of cholic acid is formed via this pathway which does not involve initial 7α-hydroxylation (11). This finding was observed in the present and previous report in each patient administered either [^{3}H]- or [^{14}C]7α-hydroxycholesterol and/or 7α-[^{3}H]hydroxy-4-cholesten-3-one. The alternative explanation for these findings is that intravenously administered labeled 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one are preferentially shunted into a chenodeoxycholic acid pathway by a selective exposure to the enzyme systems in the soluble fraction rather than in the microsomal fraction where the 12α-hydroxylation occurs. The presence of a bypass

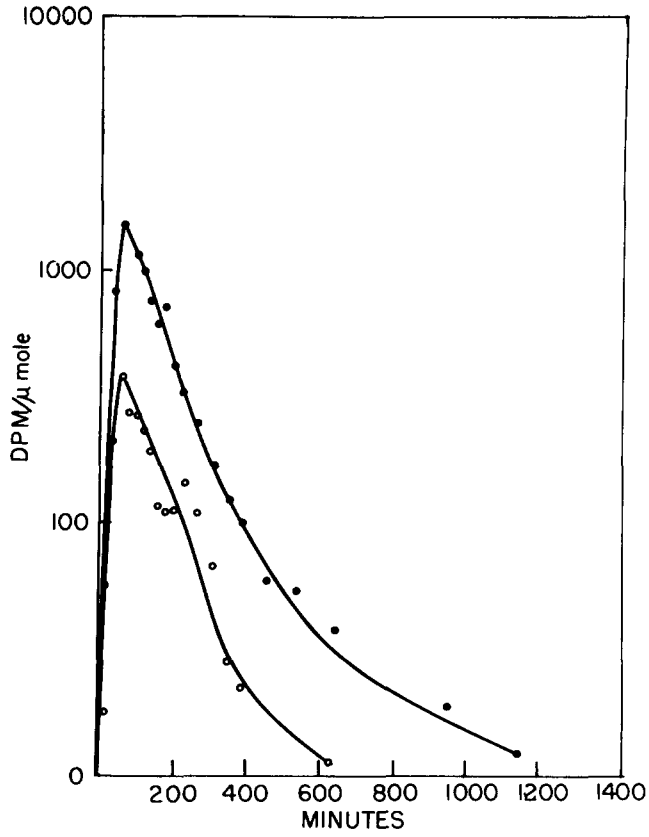

Fig. 5. Specific activity time course curves for cholic [○] and chenodeoxycholic [●] acid following the administration of 26-hydroxy-[G-^{3}H]cholesterol to patient WI.

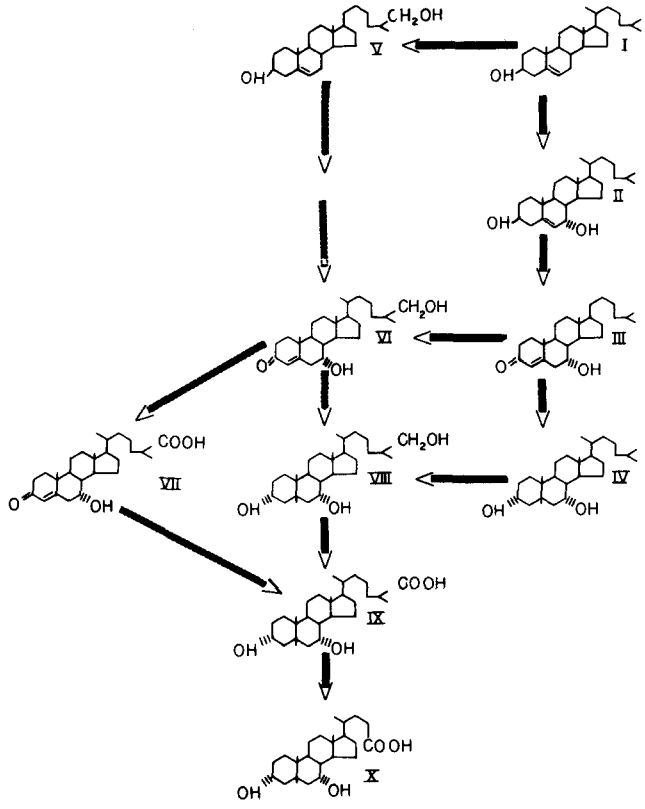

Fig. 6. Schematic representation of proposed pathways to chenodeoxycholic acid in man. I, Cholesterol; II, 7α-hydroxycholesterol; III, 7α-hydroxy-4-cholesten-3-one; IV, 5β-cholestane 3α,7α-diol; V, 26-hydroxycholesterol; VI, 7α,26-dihydroxy-4-cholesten-3-one; VII, 7α-hydroxy-3-keto-cholest-4-enoic acid; VIII, 5β-cholestane-3α,7α,26-triol; IX, 3α,7α-dihydroxy-5β-cholestanoic acid; X, chenodeoxycholic acid.

pathway is best demonstrated in patient KI who simultaneously received ^{3}H-labeled 7α-hydroxycholesterol and [^{14}C]cholesterol. The specific activities of cholic and chenodeoxycholic acids derived from [^{14}C]cholesterol were virtually identical, but were markedly different after the administration of labeled 7α-hydroxycholesterol. In patients with chronic biliary diversion, increased bile acid synthesis might theoretically exaggerate bile acid pathways of minor significance. Previous reports (7, 10–12) have provided evidence that a bypass pathway to cholic acid is also present in subjects with normal bile acid synthesis and an intact enterohepatic circuit. By contrast, labeled 7α-hydroxycholesterol administered intravenously to the rat results in identical specific activities of cholic and chenodeoxycholic acids (26), a finding which suggests that in this species an alternate pathway to cholic acid from cholesterol is not present. This discrepancy also further emphasizes the difference between rat and man in the biosynthetic routes to bile acids from cholesterol.

The present study was aimed at obtaining positive evidence for the nature of this alternate pathway to cholic acid by examining the conversion to primary bile acids of potential precursors lacking a 7α-hydroxy group. On theoretical grounds, there are three plausible substrates distal to cholesterol which could fulfill these criteria, namely 26-hydroxycholesterol, 25-hydroxycholesterol, and 12α-hydroxycholesterol. The 26-hydroxycholesterol compound was previously tested in man by Anderson, Kok, and Javitt (4) and shown to favor chenodeoxycholic acid. The data of the present report have confirmed these findings, but in addition have quantitatively suggested that this compound is very poorly converted to primary bile acids. Anderson et al. (4) obtained somewhat better but a variable recovery (10–84%) of radioactivity after administration of [^{3}H]26-hydroxycholesterol to patients with bile fistula. The compound used in their studies was 25(*R*) isomer obtained from kryptogenin while 26-hydroxycholesterol in the present report was a racemic mixture consisting of equal parts of 25-*S* and 25-*R* isomers. The discrepancy in the recovery of radioactivity between the two studies might be due to differences in the chemical structures of two administered intermediates. Some of the ^{3}H-activity might have been lost in the conversion of ^{3}H-labeled 26-hydroxycholesterol and 5-cholestene 3β,12α-diol (12α-hydroxycholesterol) into C_{24} bile acids since both of these compounds were randomly labeled with tritium (27). The results obtained with this compound do suggest that 7α-hydroxylation may be impaired if the side chain at C_{26} is hydroxylated. This is also partially inferred from the data obtained with 5β-cholestane 3α,7α,26-triol which is efficiently 12α-hydroxylated even though the side chain is 26-hydroxylated. The data obtained with 5-cholestene 3β,12α-diol indicate that very little cholic acid (10–12%) was formed from this substrate; most of the radioactivity was found on the thin layer chromatography in the deoxycholic acid zone. These findings have several implications of interest. Clearly 12α-hydroxycholesterol plays a role of minor significance in cholic acid synthesis, but it also raises the possibility that deoxycholic acid synthesis could occur in human liver if cholesterol were 12α-hydroxylated. It has also been shown in the rabbit that 5-cholestene 3β,12α-diol gives rise to deoxycholic acid and small amounts of cholic acid (28). The ability of 25-hydroxycholesterol to form cholic acid efficiently in man is currently under investigation. There is precedent for a 25-hydroxylation pathway to cholic acid in normal subjects since 5β-cholestane 3α,7α,25-triol and 5β-cholestane 3α,7α,12α,25-tetrol were found to be converted to cholic acid (9, 25). The quantitative

significance of the 25-hydroxylation pathway in man remains to be elucidated.

In the previous studies (10) it was shown that the generally accepted main precursors of chenodeoxycholic acid, 5β-cholestane 3α,7α-diol and 5β-cholestane 3α,7α,26-triol could be efficiently converted to both cholic and chenodeoxycholic acids. Furthermore, while 5β-cholestane 3α,7α-diol was converted to cholic acid and chenodeoxycholic acid in about the same proportions its predecessor 7α-hydroxy-4-cholesten-3-one preferred chenodeoxycholic acid. These observations raise questions about the purported role of 7α-hydroxy-4-cholesten-3-one as the key bifurcation compound to cholic and chenodeoxycholic acid. These unexpected findings with 7α-hydroxy-4-cholesten-3-one and 5β-cholestane 3α,7α-diol suggested the possible existence of a selective pathway to chenodeoxycholic acid which might involve the 26-hydroxylation of 7α-hydroxy-4-cholesten-3-one to produce 7α,26-dihydroxy-4-cholesten-3-one. There is evidence in support of such a reaction, since in vitro studies (2, 6) have shown that human and rat liver mitochondria can efficiently 26-hydroxylate 7α-hydroxy-4-cholesten-3-one. When 7α,26-dihydroxy-4-cholesten-3-one was tested in vivo, it was found to be very efficiently converted to bile acids, but it favored chenodeoxycholic acid over cholic acid by a wide margin (5 to 10 times) in all three bile fistula patients. Up until the present time no known bile acid intermediate, prior to 3α,7α-dihydroxy-5β-cholestanoic acid, has shown such a preference for chenodeoxycholic acid in man. The 26-hydroxylation of the side chain per se could not account for the preferential conversion to chenodeoxycholic acid since 5β-cholestane 3α,7α,26-triol was equally converted to cholic acid and chenodeoxycholic acid in the present study and also in the earlier reports (9, 11). In fact, the 5β-cholestane 3α,7α,26-triol formed more cholic acid and less chenodeoxycholic acid than the 7α,26-dihydroxy-4-cholesten-3-one. How much of the administered 7α,26-dihydroxy-4-cholesten-3-one was converted to chenodeoxycholic acid via routes other than through 5β-cholestane 3α,7α,26-triol could not be ascertained, but it would appear to be substantial. A more selective formation of chenodeoxycholic acid could only occur if 7α,26-dihydroxy-4-cholesten-3-one were converted to an intermediate which was almost exclusively converted to chenodeoxycholic acid and did not pass through 5β-cholestane 3α,7α,26-triol. Such a compound could be 7α-hydroxy-3-keto-cholest-4-enoic acid.

The presently available information from this and other recent studies on the nature of the biosynthetic pathway to chenodeoxycholic acid in man is summarized in **Fig. 6.** At least two major routes to chenodeoxycholic acid appear to exist. Both pathways proceed from cholesterol through 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one. If the latter compound is 26-hydroxylated the intermediate formed (7α,26-dihydroxy-4-cholesten-3-one) is very efficiently converted to chenodeoxycholic acid. The 5β-reduction of 7α-hydroxy-4-cholesten-3-one yields 5β-cholestane 3α,7α-diol which can then be 26-hydroxylated to form 5β-cholestane 3α,7α,26-triol. The 26-triol can also form chenodeoxycholic acid, but to a lesser extent than 7α,26-dihydroxy-4-cholesten-3-one. The quantitative significance of these pathways in vivo remains to be established. A pathway to chenodeoxycholic acid from cholesterol through 26-dihydroxycholesterol may also be present in the human liver cell, but it appears to be of only minor importance. It is of interest that this pathway could also theoretically involve the intermediate 7α,26-dihydroxy-4-cholesten-3-one.

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