Oxidoreduction of different hydroxyl groups in bile acids during their enterohepatic circulation in man

Ingemar Björkhem, Lars Liljeqvist, Klas Nilsell, and Kurt Einarsson

Departments of Clinical Chemistry, Surgery, and Medicine at the Karolinska Institute, Huddinge University Hospital, Huddinge, Sweden

Abstract The extent of oxidoreduction of the 3α-, 7α- and 12α-hydroxyl groups in bile acids during the enterohepatic circulation in man was studied with the use of [3β-^{3}H]-labeled deoxycholic acid and cholic acid, [7β-^{3}H]-labeled cholic acid, and [12β-^{3}H]-labeled deoxycholic acid and cholic acid. Each [^{3}H]-labeled bile acid was given per os to healthy volunteers, together with the corresponding [24-^{14}C]-labeled bile acid. The rate of oxidoreduction was calculated from the decrease in the ratio between ^{3}H and ^{14}C in the respective bile acid isolated from duodenal contents collected at different time intervals after administration of the labeled bile acids. The mean fractional conversion rate was found to be 0.29 day^{-1} for the 3α-hydroxyl group in deoxycholic acid (n = 2), 0.18 day^{-1} for the 12α-hydroxyl group in deoxycholic acid (n = 6), 0.09 day^{-1} for the 3α-hydroxyl group in cholic acid (n = 3), 0.05 day^{-1} for the 7α-hydroxyl group in cholic acid (n = 2), and 0.03 day^{-1} for the 12α-hydroxyl group in cholic acid (n = 2). The extent of oxidoreduction of the 12α-hydroxyl group in [12β-^{3}H]-labeled deoxycholic acid given to two patients operated with subtotal colectomy and ileostomy was markedly reduced (less than 20% of normal). The results show that the degree of oxidoreduction of bile acids in the enterohepatic circulation is relatively high and that the hydroxyl groups in deoxycholic acid are more sensitive to oxidoreduction than are the hydroxyl groups in cholic acid. The intestinal microflora seem to be essential for the oxidation, and the possibility that this oxidation is of some importance for the rate of absorption is discussed.—**Björkhem, I., L. Liljeqvist, K. Nilsell, and K. Einarsson.** Oxidoreduction of different hydroxyl groups in bile acids during their enterohepatic circulation in man. *J. Lipid Res.* 1986. **27:** 177–182.

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Bile acids are extensively metabolized during their enterohepatic circulation in man (for a review, see ref. 1). In the intestine they are subjected to dehydroxylation, deconjugation, and oxidoreduction and in the liver to reconjugation and oxidoreduction, but only to a minor extent to hydroxylation (1). Little is known, however, concerning the extent of oxidoreduction during the enterohepatic circulation. Ketonic bile acids are important constituents in feces (2–4), whereas human bile normally only contains traces of such compounds (5). We recently showed that serum from fasting human subjects, both from portal and peripheral blood, normally contains about 8–9% of monoketonic bile acids (6). Since there are oxido-reductases both in the intestine and in the liver (1), it is not possible to evaluate the total extent of oxidoreduction from the above information. Thus a 3α-hydroxyl group in a bile acid may be oxidized to yield a 3-oxo group and then reduced back to a 3α-hydroxyl group. The situation is the same for a 7α- and a 12α-hydroxyl group.

In the present work we attempted to evaluate the total extent of oxidoreduction of the different hydroxyl groups in cholic acid and deoxycholic acid with use of cholic acid labeled with ^{3}H in the 3β-, the 7β-, or in the 12β-position, and with deoxycholic acid labeled with ^{3}H in the 3β- or the 12β-position. Each ^{3}H-labeled bile acid was mixed with the corresponding ^{14}C-labeled bile acid and administered to human volunteers. Since an oxidation of a ^{3}H-labeled CH–OH group can be expected to lead to loss of ^{3}H, it was believed that the decreasing ratio between ^{3}H and ^{14}C in the corresponding bile acid isolated from duodenal contents should reflect the extent of oxidoreduction.

MATERIALS AND METHODS

Subjects

Six healthy males (age 22–32 years) and one healthy female (age 30 years) with no sign of liver or intestinal diseases volunteered for the study. A total of 15 experiments were performed on these seven subjects with an interval of at least 4 weeks between experiments in the same subject.

Patient 1 was a previously healthy 29-year-old man who in March 1984 had intermittent diarrhea with passage of blood and mucous. During the next 2 months the symptoms progressed and he was hospitalized. A severe ulcera-

Abbreviations: FCR, fractional conversion rate.

tive colitis was diagnosed. As medical therapy failed, he was operated on with subtotal colectomy and ileostomy in May 1984. He had a rapid recovery with an increase of weight of 10 kg in 3 months. The present study was performed 5 months after surgery.

Patient 2 was an otherwise healthy 46-year-old man who had ulcerative proctitis in 1968. In 1976 it turned into a pancolitis and he had a severe exacerbation in 1977. At that time the disease responded well to intensive medical treatment. In September 1984 he had a new exacerbation that progressed into an acute fulminating status despite intensive medical treatment. He was operated on in October 1984 with subtotal colectomy and ileostomy and had a rapid recovery. He was in excellent condition, had regained his weight, and had normal laboratory examinations when the present study was undertaken 2 months after surgery.

Ethical aspects

The ethical aspects of the present study were approved by the Ethical Committee of the Karolinska Institute at Huddinge Hospital.

Methods

[3β-^{3}H]-Labeled cholic acid was prepared by oxidation of methylated cholic acid (7) to yield methyl 7α,12α-dihydroxy-3-oxo-5β-cholanoic acid. The latter compound was purified by preparative thin-layer chromatography, using system S 11 (8). The ketonic steroid was dissolved in 0.5 ml of methanol, and a few grains of ^{3}H-labeled sodium borohydride were added (obtained from the Radiochemical Center, Amersham, England). After 30 min at room temperature, excess unlabeled sodium borohydride was added and, after an additional 30-min period, the reduced material was extracted by ethyl ether from an acidified aqueous phase. The [3β-^{3}H]-labeled methyl cholic acid was purified by thin-layer chromatography, using system S 11 (8). The material obtained was pure as judged by thin-layer chromatography and gas–liquid chromatography (as the methyl ester trimethylsilyl derivative, using a 1.5% SE-30 column) and had a specific radioactivity of 210 × 10^6 cpm/mg. The radiochemical purity was tested by oxidation of part of the [3β-^{3}H]-labeled methyl cholic acid together with methyl[24-^{14}C]-cholic acid as above to yield methyl 7α,12α-dihydroxy-3-oxo-5β-cholanoic acid. The purified 3-oxo-steroid had lost 96% of the ^{3}H-label as judged from the ^{3}H/^{14}C ratio. The free [3β-^{3}H]-labeled cholic acid was prepared from the corresponding methyl ester by alkaline hydrolysis.

[7β-^{3}H]-Labeled cholic acid was prepared by oxidation of methylated cholic acid with N-bromosuccinimide to yield methyl 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (9). The latter compound was purified by thin-layer chromatography and reduced with ^{3}H-labeled sodium borohydride as above. The purified product, methyl [7β-^{3}H]-cholic acid, was pure as judged by thin-layer chromatography and gas–liquid chromatography and had a specific radioactivity of 240 × 10^6 cpm/mg. The radiochemical purity was tested by oxidation of part of the material together with methyl [24-^{14}C]cholic acid as above to yield methyl 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid. The purified 7-oxo-steroid had lost more than 95% of the ^{3}H as judged from the ^{3}H/^{14}C ratio. The free [7β-^{3}H]-labeled cholic acid was prepared from the methyl ester as above.

[12β-^{3}H]-Labeled cholic acid was prepared biosynthetically from [12β-^{3}H]-labeled deoxycholic acid (see below) utilizing the high capacity of rat liver to 7α-hydroxylate deoxycholic acid. Part of the [12β-^{3}H]-labeled deoxycholic acid described below was administered intraperitoneally to a bile fistula rat under the conditions previously described (10). After alkaline hydrolysis, the [12β-^{3}H]-labeled cholic acid was isolated from the bile (10) and purified by thin-layer chromatography as the methyl ester as described above. The material had a specific radioactivity of 28 × 10^6 cpm/mg and was pure as judged by thin-layer chromatography and gas–liquid chromatography. The free [12β-^{3}H]-labeled cholic acid was prepared as above.

[12β-^{3}H]-Labeled deoxycholic acid was prepared by oxidation of the 12α-hydroxyl group in methyl deoxycholic acid with chromic acid as described by Bergström and Haslewood (11) to yield methyl 3α-hydroxy-12-oxo-5β-cholanoic acid. The latter compound was reduced with ^{3}H-labeled sodium borohydride and the resulting [12β-^{3}H]-labeled methyl deoxycholic acid was purified by thin-layer chromatography as above. The isolated material was pure as judged by thin-layer chromatography and gas–liquid chromatography and had a specific radioactivity of 83 × 10^6 cpm/mg. The radiochemical purity was tested by oxidation of part of the material together with [24-^{14}C]-methyl deoxycholic acid as above to yield methyl 3α-hydroxy-12-oxo-5β-cholanoic acid. The purified 12-oxosteroid had lost more than 90% of the ^{3}H as judged from the ^{3}H/^{14}C ratio. The free steroid was prepared from the methyl ester by alkaline hydrolysis.

[3β-^{3}H]-Labeled deoxycholic acid was prepared by oxidation of the 3α-hydroxyl group in methyl deoxycholic acid (7) followed by reduction of the purified 3-oxosteroid with ^{3}H-labeled sodium borohydride. The purified product had a specific radioactivity of 150 × 10^6 cpm/mg and was pure as judged by thin-layer chromatography and gas–liquid chromatography. The radiochemical purity was better than 95% as judged by the ^{3}H/^{14}C ratio after re-oxidation of the 3α-hydroxyl group after addition of methyl [24-^{14}C]deoxycholic acid. The free [3β-^{3}H]-labeled steroid was prepared from the methyl ester by alkaline hydrolysis.

[24-^{14}C]-Labeled cholic acid and deoxycholic acid were obtained from New England Nuclear (Dreieichenhain, West Germany) and had a specific radioactivity of 220 × 10^6

cpm/mg. The compounds were purified (as methyl esters) by thin-layer chromatography as above before use.

Administration of the ^{3}H- and ^{14}C-labeled bile acids and collection of bile. Each ^{3}H-labeled bile acid was mixed with the corresponding [24-^{14}C]-labeled bile acid to give a $^{3}H/^{14}C$ ratio between 1.5 and 5. The minimum amount of ^{14}C given was 2.0 × 10^{6} cpm and the maximum was 5.0 × 10^{6} cpm. The minimum amount of ^{3}H given was 4 × 10^{6} cpm and the maximum was 9 × 10^{6} cpm. The weight of labeled material given never exceeded 150 μg. The labeled bile acid mixture was given as a sodium salt in water in the evening. Four samples of fasting duodenal bile were collected from each subject after about 12, 36, 60, and 84 hr.

In one case the $^{3}H/^{14}C$-labeled bile acid was administered intravenously (cf. Results). In this case the ethanolic solution of the mixture was passed through a Millex 0.22-μm filter. Immediately prior to infusion, the ethanolic solution was mixed with a sterile solution of sodium chloride in water (0.9%, w/v) and the mixture was slowly infused intravenously.

Cholecystokinin (40 Ivy-units) was administered intravenously, and about 5 ml of concentrated duodenal bile was obtained through a thin polyvinyl tube with the collecting orfice positioned fluoroscopically at the ampulla of Vater.

Isolation of bile acids and assay of radioactivity. The bile samples were hydrolyzed with 1 M KOH at 110°C for 12 hr. After acidification, the deconjugated bile acids were extracted with diethyl ether and methylated with diazomethane. The methyl esters of cholic and deoxycholic acids were isolated from the remaining material by thin-layer chromatography using system S8 and system S11, respectively (8). The purity of the isolated bile acids was checked by gas–liquid chromatography as above. Radioactivity was measured with an Intertechnique SL 30 liquid scintillation spectrometer, using Luma gel (LUMAC, BV, The Netherlands) as scintillation fluid. Under the conditions employed, the counting efficiency was 71% for ^{14}C and 49% for ^{3}H. No corrections for quenching were found to be necessary. In the early part of the study the isolated bile acids were diluted with the corresponding methylated unlabeled bile acids and crystallized three times from acetone–water and methanol–water mixtures. Since such crystallization never changed the $^{3}H/^{14}C$ ratio significantly, the crystallization was omitted in the latter part of the study.

RESULTS

Experiments with healthy subjects

As seen in **Fig. 1**, there was a rapid decrease in the $^{3}H/^{14}C$ ratio in isolated cholic acid during the first 12 hr after oral administration of [3β-^{3}H]-labeled cholic acid and [7β-^{3}H]-labeled cholic acid. Between 12 and 84 hr, however, the decrease in ratio was considerably slower. This general pattern was seen in all three subjects receiving [3β-^{3}H]cholic acid and in the two subjects receiving [7β-^{3}H]cholic acid. Since it was believed that the rapid decrease during the first 12 hr may have been due to the oral administration, one of the subjects who had received [3β-^{3}H]cholic acid per os also received the same compound intravenously 2 months later. As seen in Fig. 1, there was no early rapid decrease after the intravenous administration. Furthermore, the fractional conversion rate (FCR) calculated from the decrease in ratio after the intravenous administration (FCR = 0.08 day^{-1}) was essentially the same as that obtained between 12 and 84 hr after the oral administration (FCR = 0.10 day^{-1}). In view of this, the first rapid decrease in ratio during the first 12 hr was not taken into account when the FCR was calculated.

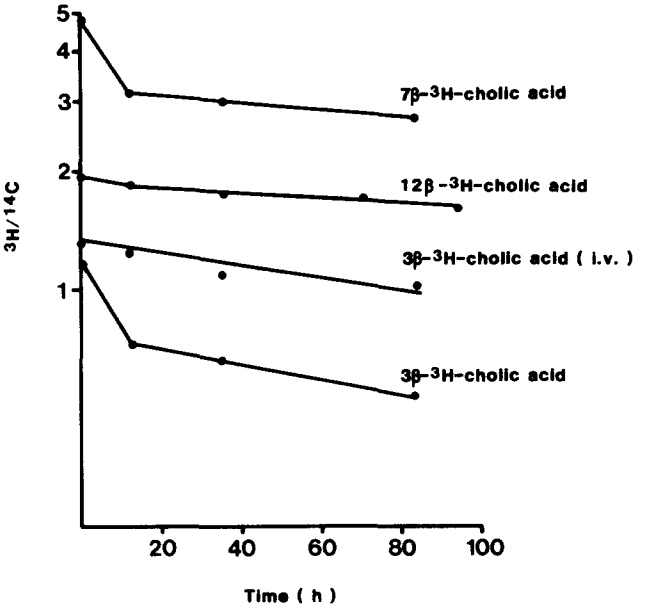

Fig. 1. Decay of $^{3}H/^{14}C$ ratio in cholic acid isolated from duodenal bile after oral administration of [7β-^{3}H]- + [24-^{14}C]cholic acid, [12β-^{3}H]- + [24-^{14}C]cholic acid, and after oral and intravenous administration of [3β-^{3}H]- + [24-^{14}C]cholic acid.

The FCR for the 3α-hydroxyl group in cholic acid was calculated to be 0.09, 0.10, and 0.09 day^{-1}, respectively, in the three different subjects studied. The FCR for the 7α-hydroxyl group in cholic acid was found to be 0.05 day^{-1} in both subjects studied. The FCR for the 12α-hydroxyl group in cholic acid was found to be 0.05 day^{-1} in one subject (Fig. 1) and 0.00 day^{-1} in another.

As seen in **Fig. 2**, the decrease in the $^{3}H/^{14}C$ ratio in deoxycholic acid after administration of [3β-^{3}H]- and [12β-^{3}H]-labeled deoxycholic acid was considerably greater than that obtained with [3β-^{3}H]- and [12β-^{3}H]-labeled cholic acid. There was no early decrease during the first 12 hr in this case. The FCR for the 3α-hydroxyl group in

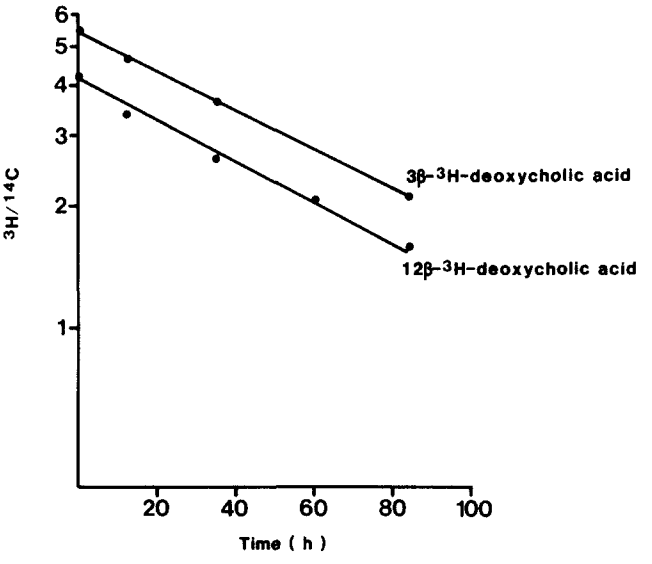

Fig. 2. Decay of $^3H/^{14}C$ ratio in deoxycholic acid isolated from duodenal bile after oral administration of [12β-^{3}H]- + [24-^{14}C]deoxycholic acid and [3β-^{3}H]- + [24-^{14}C]deoxycholic acid.

deoxycholic acid was found to be 0.34 and 0.23 day^{-1}, respectively, in the two subjects studied. The FCR for the 12α-hydroxyl group in deoxycholic acid was found to be 0.27, 0.17, 0.26, 0.14, 0.16, and 0.08 day^{-1}, respectively, in the six subjects studied (mean = 0.18 day^{-1}). The results of the experiments with the healthy subjects are summarized in **Table 1** and **Fig. 3**.

Experiments with patients 1 and 2

After administration of [12β-^{3}H]-labeled deoxycholic acid to the two patients, the decrease in the $^3H/^{14}C$ ratio was considerably slower than was the case in the healthy subjects. The FCR for the 12α-hydroxyl group was calculated to be 0.02 and 0.05 day^{-1}, respectively.

DISCUSSION

The present work shows that there is a relatively high degree of oxidoreduction of hydroxyl groups in cholic acid and deoxycholic acid during the enterohepatic circulation. Thus the FCR for the three hydroxyl groups in cholic acid (FCR of 3α-OH + FCR of 7α-OH + FCR of 12α-OH) was found to be about 0.2 day^{-1}, whereas the FCR for the two hydroxyl groups in deoxycholic acid (FCR of 12α-OH + FCR of 3α-OH) was about 0.5 day^{-1}. In view of the fact that the concentration of ketonic bile acids is high in feces and low in bile, it seems likely that the oxidation occurs mainly in the intestine. Attempts to evaluate the relative roles of the liver and intestine were made in the present work by studying the oxidoreduction of the 12α-hydroxyl group in [12β-^{3}H]-labeled deoxycholic acid in two patients with subtotal colectomy and ileostomy. In these experiments there should have been very little exposure of the labeled bile acid to the intestinal microflora. Since the FCR for the oxidoreduction of the 12α-hydroxyl group decreased to less than 20% of that obtained in the experiments with the healthy subjects, it must be concluded that the intestine is of major importance. The high capacity of the liver to reduce trioxocholanoic acid to cholic acid (12) and 7-oxolithocholic acid to chenodeoxycholic acid (13, 14) is also in accord with the contention that the oxidative step mainly occurs in the intestine. If the intestine is the limiting factor, increased oxidoreduction can be expected under conditions of increased exposure of the bile acids to the intestinal microflora. Bacterial overgrowth in the small intestine and increased cycling rates in the enterohepatic circulation may thus be conditions associated with a higher degree of oxidoreduction of bile acids. The finding by Hepner et al. (5) that ketonic bile acids composed 7–15% of the bile acid pool in cholecystectomized patients is in agreement with this. In cholecystectomized patients there is an increased recycling of bile acids and an increased formation of secondary bile acids (for a review, see ref. 15).

The FCR for oxidation of the different hydroxyl groups was calculated from the decrease in the $^3H/^{14}C$ ratio obtained 12–84 hr after the administration of the labeled bile acids. The more rapid decrease obtained in most experiments during the first 12 hr was thus not taken into account. It is possible that the early rapid oxidoreduction is associated with the first enterohepatic cycle of the bile acids. Since the bile acids given were unconjugated and

TABLE 1. Results of experiments performed with healthy subjects (oral administration)

Subject	3α-Hydroxyl Group in Cholic Acid	7α-Hydroxyl Group in Cholic Acid	12α-Hydroxyl Group in Cholic Acid	3α-Hydroxyl Group in Deoxycholic Acid	12α-Hydroxyl Group in Deoxycholic Acid
			FCR (day^{-1})		
1	0.10		0.00	0.34	0.17
2	0.09	0.05			0.27
3	0.09	0.05			0.26
4					0.14
5				0.23	0.16
6					0.08
7			0.05		

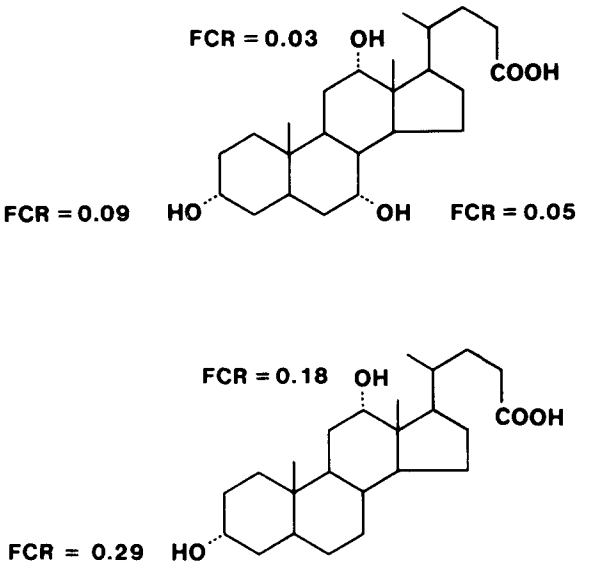

Fig. 3. Mean fractional conversion rate (FCR) of oxidoreduction of the different hydroxyl groups in the bile acids studied (healthy subjects).

can be expected to be conjugated after the first passage through the liver, it is possible that the conjugation may be of importance for the bacterial oxidoreduction in the intestine.

The composition of the bile acid pool size may also be of some importance for this effect. Thus the endogenous pool of unconjugated bile acids should be small as compared to the pool of conjugated bile acids, and the intestinal oxidoreductases are thus initially exposed to a relatively small pool of unconjugated bile acids with a high specific radioactivity. No attempts were made, however, to correlate the rapid initial FCR to the composition of the bile acid pool in the different subjects.

It should be emphasized that the present technique gives a minimum figure for the extent of oxidoreduction, and there are two factors that may cause an underestimation. There may be an isotope effect in the oxidation of a hydroxyl group adjacent to a ^{3}H-label. Most probably, if present, such an isotope effect is small. In preliminary experiments using [3β-^{3}H]-labeled bile acids as substrate for a bacterial 3α-hydroxysteroid dehydrogenase, no significant isotope effect was found (K_H/K_T = 0.96–1.03 in different experiments). There may be a transfer of ^{3}H from the labeled bile acid to the cofactor of a hydroxysteroid dehydrogenase in an oxidative step and a subsequent utilization of the same labeled cofactor by the hydroxysteroid dehydrogenase in a reductive step. This would lead to retention of tritium in the bile acid in spite of an oxidoreduction. A similar mechanism has, in fact, been demonstrated in the conversion of cholesterol into coprostanol by fecal microorganisms (16). Thus [3α-^{3}H]-cholesterol was converted into coprostanol in this system with retention of some of the ^{3}H in spite of an intermediate oxidoreduction of the 3β-hydroxyl group. Shefer and collaborators (17) have shown that 3β-isomers of bile acids (isobile acids) with a ^{3}H-label in the 3α-position were converted into the corresponding 3α-hydroxy bile acids in the liver with retention of most of the ^{3}H-label. A transfer of ^{3}H from the 3β-hydroxysteroid to the cofactor of the 3β-hydroxysteroid dehydrogenase and a subsequent utilization of the same labeled cofactor by the 3α-hydroxysteroid dehydrogenase may be the explanation for this finding.

We can not exclude that such a mechanism may be operating to some extent also in the metabolism of [3β-^{3}H]-labeled 3α-hydroxysteroids, [7β-^{3}H]-labeled 7α-hydroxysteroids, and [12β-^{3}H]-labeled 12α-hydroxysteroids. On the other hand, if such a mechanism is of importance, a transfer of ^{3}H could be expected to occur from ^{3}H-labeled cholic acid to chenodeoxycholic acid and from ^{3}H-labeled deoxycholic acid to cholic acid and chenodeoxycholic acid. Little or no such transfer was observed in our experiments.

The high extent of oxidoreduction of the hydroxyl groups in deoxycholic acid is in accord with the finding of high concentrations of ketonic deoxycholic acid and isodeoxycholic acid in human feces (2). It should be pointed out that in the present work we have only measured the extent of oxidoreduction of the bile acids retained in the enterohepatic circulation and no attempts were made to study the oxidoreduction of the fecal bile acids. It is possible that the more nonpolar ketonic bile acids formed in the intestine are less readily absorbed and bacterial oxidation may be an important determinant for the degree of absorption of bile acids and thus also for their turnover rates. Studies on this subject are in progress.

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