METABOLIC STUDIES OF BILE ACIDS. XLV[1]. THE TRANSFORMATION OF 3β,-7α-DIHYDROXYCHOL-5-ENIC-24-^{14}C ACID TO CHENODEOXYCHOLIC ACID IN THE RAT. THE SIGNIFICANCE OF THE C-7α-HYDROXYL GROUP IN BILE ACID FORMATION.

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ABSTRACT

When 7α,12α-dihydroxy-3-oxocholanic-12β-^{3}H acid and 7α--hydroxy-3-oxocholanic-7β-^{3}H acid were administered to rats furnished with bile fistulas, cholic acid and chenodeoxycholic acid, respectively, were excreted in their bile. In addition, 3β,7α-dihydroxychol-5-enic-24-^{14}C acid was converted to chenodeoxycholic acid. The significance of the C-7α-hydroxyl group in bile acid formation from cholesterol is discussed.

It is well known that cholic (3α,7α,12α-trihydroxycholanic) and chenodeoxycholic (3α,7α-dihydroxycholanic) acids are the primary bile acids in rat bile. In the *in vivo* experiments of the bile acid formation in rat, several kinds of sterol as well as bile acid derivatives were tested,[2,3] some of which have proved to be converted into cholic acid and/or chenodeoxycholic acid.

Although some hydroxylated coprostane derivatives including the corresponding C-27-oic acids are assumed to be probable intermediary products of the bile acid formation, there has not been obtained any clear-cut explanation how the rearrangements of the nuclear part of cholesterol molecule, especially those of Rings A and B, are carried out to give the specific configuration of the bile acid molecule. Based upon the findings that 7α-hydroxycholesterol is not only converted into the primary bile acids but also dehydrogenated to a 3-oxo compound, probably cholest-4-en-7α-ol-3-one, by the specific enzyme (system) in rat liver, we have advanced the hypothesis that the last named 3-oxo compound be an obligatory intermediary product in the bile acid biosythesis.[4,5] Recently the experimental data in favor of our assumption have been accumulated.[6,7]

In the present paper the metabolism of a C-24 analogue of 7α-hydroxycholesterol, 3β,7α-dihydroxychol-5-enic-24-^{14}C acid and those of the tritiated C-3 oxo-bile acids having C-7α hydroxyl group are studied in order to get more precise information upon the nuclear rearrangements, especially the C-3 epimerization, in the bile acid formation from cholesterol.

MATERIALS

3β,7α-Dihydroxychol-5-enic-24-^{14}C Acid. As will be reported elsewhere, the unlabeled sample of the title acid was prepared from methyl 3β-acetoxychol-5-enate by the method analogous to that of preparing 7α-hydroxycholesterol from cholesterol.[8] M.p. 213°C, $[\alpha]_D$-87°; methyl ester,

m.p. 161-163°, $[\alpha]_D$-177°.

The corresponding 24-^{14}C acid was afforded by the same method as described above from methyl 3β-hydroxychol-5-enate-24-^{14}C, which was prepared from hyodeoxycholic acid-24-^{14}C,[9,10] and purified by reversed phase partition chromatography.[11] Crystallized together with the unlabeled authentic sample described above, it showed specific activity of 1.077×10^5 c.p.m./mg.

7α,12α-Dihydroxy-3-oxocholanic-12β-^{3}H Acid. Methyl 3α,7α-dihydroxy-12-oxocholanate, prepared according to the method described by Fieser and Rajagopalan,[12] was reduced by natrium borohydride-^{3}H in aqueous dioxane. The resulting methyl cholate-12β-^{3}H was converted through methyl 7α,12α-diacetoxy-3α-hydroxycholanate into the 3-oxo compound by the method reported by Haslewood.[13] Reversed phase partition chromatography of the product gave a pure sample of 7α,12α-dihydroxy-3-oxocholanic-12β-^{3}H acid; m.p. 172°C; specific activity: 5.03×10^4 c.p.m./mg.

7α-Hydroxy-3-oxocholanic-7β-^{3}H Acid. This tritiated acid was prepared from chenodeoxycholic-7β-^{3}H acid by the method analogous to that described above.[11] At first an unlabeled sample of 7α-hydroxy-3-oxocholanic acid was prepared as follows. Methyl diacetyl chenodeoxycholic acid (0.80 gm; m.p. 129°C) was refluxed in 20 ml of methanol containing 0.85 ml of concentrated hydrochloric acid for 3 hours. The reaction mixture was diluted with water, and extracted twice with ether, washed with water, dried over natrium sulfate and then evaporated to a syrup. The syrup thus obtained was dissolved in 10 ml of acetic acid and oxidized carefully with 2.5 ml of a 10 per cent solution of chromic anhydride in acetic acid. After being left at 50°C for 80 minutes, the reaction mixture was diluted with water, and extracted with ether, washed with water, a 2 per cent solution of natrium carbonate and again water successively. The ether layer was dried over natrium sulfate and evaporated to dryness. Several recrystallizations from aqueous metha-

nol afforded 585 mg of methyl 7α-acetoxy-3-oxocholanate; m.p. 113-115°C (Hauser et al.[14]: m.p. 115-117°C); Zimmermann's reaction positive; I.R.: 1750, 1250 cm^{-1}. The ester (250 mg) thus obtained was refluxed with 10 ml of a 5 per cent solution of kalium hydroxide in aqueous ethanol for one hour. The acidified hydrolyzate was extracted with ether and the evaporated extract was subjected to reversed phase chromatography on a column of hydrophobic celite (25 g) with stationary phase (chloroform-heptane 9:1 v/v; 20 ml). The elution with aqueous methanol (60 per cent) gave 164 mg of material within the 400-600 ml elution volumes. Recrystallizations of this material from aqueous methanol afforded 86 mg of 7α-hydroxy-3-oxocholanic acid; m.p. 85-87°C, Zimmermann's reaction positive; I.R.: 3450, 1713 cm^{-1}.

7α-Hydroxy-3-oxocholanic-7β-^{3}H acid was prepared from the tritiated methyl chenodeoxycholate just by the same method as mentioned above. The latter compound was afforded by reduction of 3α-hydroxy-7-oxocholanic acid[15] with natrium borohydride-^{3}H. Specific activity of the tritiated 3-oxo compound: 1.78×10^{5} c.p.m./mg.

EXPERIMENTAL

White male rats weighing about 150 g were furnished with bile fistulas as usual. After 24 hours' canulation, the animals were injected intraperitoneally with the respective samples of the isotope-labeled bile acids described above, each of which was dissolved in 0.5 ml of physiological saline (natrium salt). The fistula-bile samples of the individual animals were collected in the vessels each containing 30 ml of ethanol and filtered. Each ethanolic solution was evaporated to dryness, and saponified with a 15 per cent solution of kalium hydroxide at 130°C for 3 hours. The acidified hydrolyzate was extracted with ether as usual and then the evaporated extract was subjected to reversed phase partition chromatography.[11]

RESULTS

I. METABOLISM OF 3β,7α-DIHYDROXYCHOL-5-ENIC-24-^{14}C ACID

^{14}C-3β,7α-Dihydroxychol-5-enic acid (1.98 mg) was injected intraperitoneally into a rat furnished with a bile fistula, and all of the radioactivity administered (107 per cent) was recovered in the bile sample collected within 20 hours. Saponified and chromatographed as described above, this bile sample developed two radioactive peaks corresponding to the trihydroxylated and dihydroxylated bile acid fractions respectively, as shown in Fig. 1.

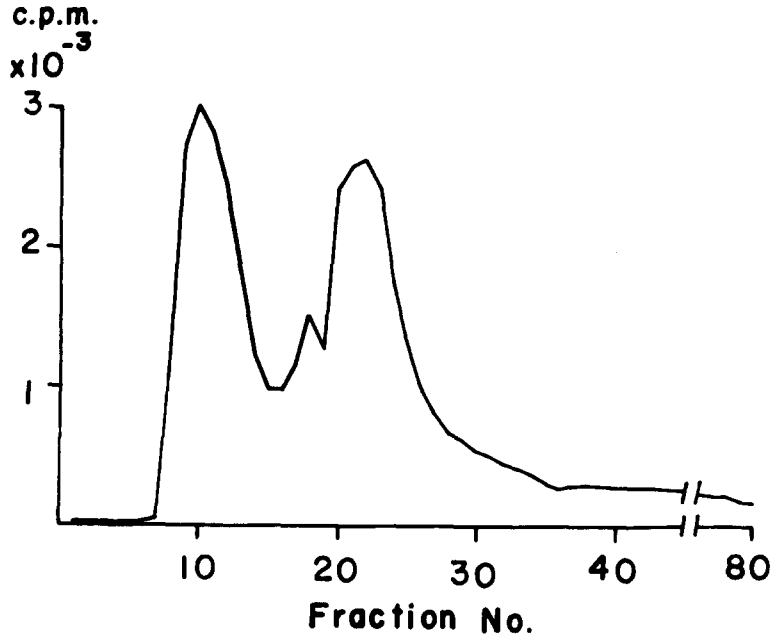

Fig. 1 Chromatography of the bile acids extracted from the hydrolyzed fistula bile of the rat which received 3β,7α-dihydroxychol-5-enic-24-^{14}C acid.

The fractions corresponding to the second peak were diluted with 80.0 mg of authentic chenodeoxycholic acid.

After the radioactivity was estimated (125 c.p.m./μ mole), the mixture was methylated with diazomethane as usual and treated with acetic anhydride-pyridine on a boiling water bath for 2 hours. The reaction mixture was worked up as usual and the resulting diacetyl methyl chenodeoxycholate was crystallized from methanol-water. Recrystallized further from methanol-water and acetone-water, it showed constant specific activity throughout the recrystallization (Table I).

Table I

Isotope dilution experiment of the extract obtained from the second peak of Fig. 1; chenodeoxycholic acid* added was converted into methyl diacetate.

No. of recrystal-lization	Solvent	Amount (mg)	Specific activity (c.p.m./μ mole)
1st	Methanol-water	44.0	109
2nd	"	34.3	106
3rd	"	26.0	106
4th	"	19.5	103
5th	Acetone-water	4.9	107

* The specific activity of the extract mixed with unlabeled chenodeoxycholic acid (80 mg) was found to be 125 c.p.m./μ mole.

From the data shown in the table, it is calculated that approximately 84 per cent of the radioactivity of the fractions corresponding to the second peak was contained in chenodeoxycholic acid molecule. The remaining part of the radioactivity (16 per cent) in these fractions was not further examined, but since the two peaks in Fig. 1

overlapped each other, it could be reasonably assumed that a small amount of trihydroxycholanic acid or acids was contaminated with the dihydroxylated bile acid fraction, the major part of which, anyway, is chenodeoxycholic acid.

The extract from the fractions of the first peak was diluted with 83.0 mg of authentic cholic acid and recrystallized repeatedly from aqueous methanol and ethyl acetate. The radioactivity was diminished completely by several recrystallizations (Table II). It is clearly shown that this peak does not correspond to cholic acid but it is very

Table II

Isotope dilution of the extract obtained from the first peak of Fig. 1; cholic acid (83 mg) was added.

No. of recrystallization	Solvent	Amount (mg)	Specific activity (c.p.m./μ mole)
	(Before recrystallization)	83.0	107
2nd	Methanol-water	54.9	38
4th	Ethyl acetate	41.6	9
6th	"	23.9	5
7th	"	19.4	0

probable that this corresponds to that of a muricholic acid derived from chenodeoxycholic acid-^{14}C as was the case reported by Matschiner et al.[16]

II. METABOLISM OF THE 3-OXO-BILE ACIDS IN RATS

Tritiated Cholic Acid from 7α,12α-Dihydroxy-3-oxocholanic-12β-^{3}H Acid. The tritiated 7α,12α-dihydroxy-3-oxocholanic acid mentioned above (2.0 mg) was injected

intraperitoneally into a bile-fistulated rat. During 20 hours, 72 per cent of the injected tritium was excreted in the bile collected. The free bile acid mixture obtained from this bile sample was chromatographed to give only one radioactive peak.

This peak was coincident with the titration curve of cholic acid and the fractions corresponding to this peak were collected and extracted with ether on acidification. The ether extract containing 49 mg of dried material was mixed with 75 mg of authentic cholic acid (total weight, 124 mg; specific activity of the mixture, 51 c.p.m./μ mole). The diluted sample was recrystallized repeatedly from methanol-water and ethyl acetate, and specific activities (c.p.m./μ mole) of the recrystallized samples remained constant, as shown in Table III. This radioactive acid was further identified by preparation of methyl ethoxycarbonyl cholate.[17] From these results it is clearly shown that the tritium excreted in the bile was present exclusively in cholic acid molecule.

Tritiated Chenodeoxycholic Acid from 7α-Hydroxy-3-oxocholanic-7β-^{3}H Acid. The tritiated 7α-hydroxy-3-oxocholanic acid (2.0 mg) was injected intraperitoneally into a bile-fistulated rat. The bile collected during 20 hours contained 60 per cent of the added tritium. The saponified bile was analyzed by chromatography as mentioned above. As shown in Fig. 2, the radioactivity is distributed in two peaks, one appearing slightly faster than that of cholic acid, and the other corresponding quite well to

Table III

Isotope dilution of the metabolite of 7α,12α-dihydroxy-3-oxocholanic-12β-^{3}H acid; cholic acid (75 mg) was added.

No. of recrystallization	Solvent	Amount (mg)	Specific activity (c.p.m./μ mole)
	Free acid		
	(Before recrystallization)	124	51
2nd	Methanol-water	67.2	51
4th	"	48.9	52
6th	Ethyl acetate	25.1	47
8th	"	13.2	55
	Methyl ethoxycarbonyl cholate		
2nd	Isopropyl ether-hexane	2.9	54

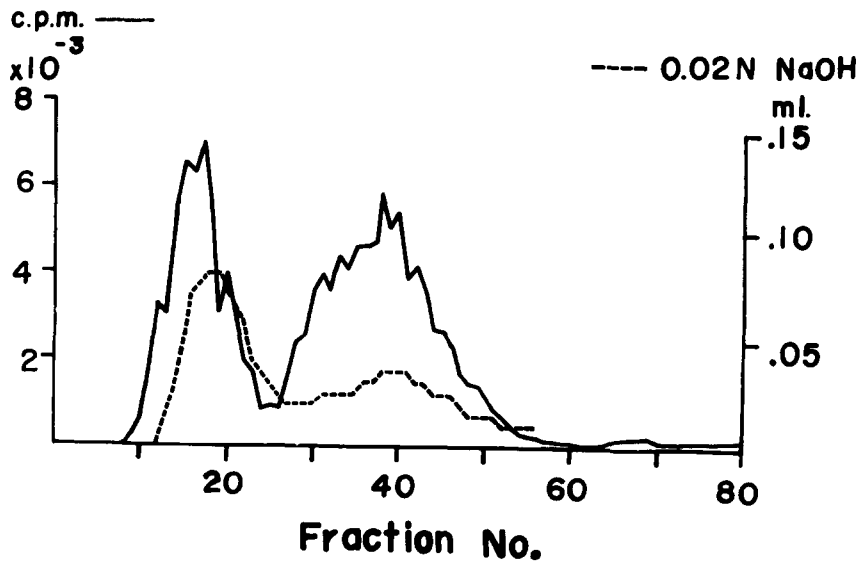

Fig. 2 Chromatography of the bile acids extracted from the fistula bile of the rat which received 7α-hydroxy-3-oxocholanic-7β-^{3}H acid.

that of chenodeoxycholic acid.

The fractions corresponding to the second peak were collected and the extract obtained as mentioned above was diluted with 100 mg of authentic chenodeoxycholic acid. After the radioactivity was estimated (593 c.p.m./μ mole), the mixture was converted into methyl diacetate as described above and was recrystallized from methanol-water or methanol alone. Specific activity of each recrystallized sample remained constant and that of the initial mixture was not decreased by several recrystallizations as shown in Table IV. The results clearly show that almost all of the tritium corresponding to the second peak was contained in chenodeoxycholic acid molecule.

Table IV

Isotope dilution of the extract obtained from the second peak of Fig. 2; chenodeoxycholic acid* was added and converted into methyl diacetate.

No. of recrystallization	Solvent	Amount (mg)	Specific activity (c.p.m./μ mole)
1st	Methanol-water	105.4	554
2nd	Methanol	58.1	560
3rd	Methanol-water	46.0	594
4th	"	33.4	583
5th	"	30.0	601

* The specific activity of the extract mixed with unlabeled chenodeoxycholic acid (100 mg) was found to be 593 c.p.m./μ mole.

The fractions corresponding to the first radioactive peak was collected, extracted with ether on acidification

and mixed with authentic cholic acid (50 mg), specific activity of the mixture being 4.39×10^3 c.p.m./μ mole. The radioactivity was rapidly diminished by repeated recrystallizations from methanol-water and ethyl acetate (Table V). This peak does not correspond to that of cholic acid, but probably to that of a muricholic acid,[16] the identification of which is under investigation.

Table V

Isotope dilution of the extract obtained from the first peak of Fig. 2; cholic acid (50 mg) was added.

No. of recrystallization	Solvent	Amount (mg)	Specific activity (c.p.m./μ mole)
	(Before recrystallization)	50.0	4.39×10^3
2nd	Methanol-water	30.2	0.12 "
3rd	"	21.5	0.07 "
4th	Ethyl acetate	16.2	0.06 "

DISCUSSION

Bergström et al.[2] are of opinion that in the course of the bile acid biosynthesis, the nuclear transformation of cholesterol precedes the degradation of its side chain, and that 7α-hydroxycholesterol is the immediate metabolite of cholesterol.[18] The findings, that 3α,7α-dihydroxy- and 3α,7α,12α-trihydroxycoprostane are converted efficiently into chenodeoxycholic and/or cholic acids,[19,20] seem to be in favor of the assumption of the Swedish workers. The bile acid formation, however, involves several kinds of yet unsolved problems. As for the transformation of cholesterol

there are three steps to be elucidated: 1) hydroxylation at the positions of C-7 and/or C-12, 2) epimerization of 3β-hydroxyl group, and 3) reduction of the C-5 double bond.

The present observation that a 7α-hydroxycholesterol analogue, 3β,7α-dihydroxychol-5-enic acid, was converted efficiently into chenodeoxycholic acid but not into cholic acid suggests that among the courses by which chenodeoxycholic acid is formed from 7α-hydroxycholesterol, there might exist a pathway in which the side chain is degradated before any of the nuclear transformations, and that 3β,7α-dihydroxychol-5-enic acid is an intermediary product to chenodeoxycholic acid.

It is the well known fact that the animals of some species excrete chenodeoxycholic acid in bile as the major metabolite of cholesterol, the excretion of cholic acid being demonstrated scarcely or much less than that of chenodeoxycholic acid. Such a metabolic course as mentioned above, therefore, might be the main route of the cholesterol degradation in the liver of these animals.

It is an interesting fact that C-3 hydroxyl group not only of the primary bile acids but also that of the most secondary ones with very few exceptions is of 3α-configuration. The results of the experiments reported here indicate that both 3-oxocholanic acid derivatives, each having C-7α hydroxyl group, i. e., 7α,12α-dihydroxy-3-oxo- and 7α-hydroxy-3-oxo-cholanic acids, were exclusively reduced to 3α-hydroxy-compounds, cholic and chenodeoxycholic acids

respectively, and furthermore, that a C-24 analogue of the 7α-hydroxycholesterol, 3β,7α-dihydroxychol-5-enic acid was actually transformed into chenodeoxycholic acid.

On the other hand, it has been demonstrated in our laboratory that both 3α- and 3β-hydroxylated compounds were isolated as the respective metabolites of 3,7,12-trioxocholanic,[21,22] 3,12-dioxocholanic[23] and 3-oxocholanic[24] acids in the in vivo as well as in vitro experiments. It is interesting to note that 3-oxochol-4-enic-24-^{14}C acid was transformed in rat not only into two epimers of 3-hydroxy-5β-cholanic acid but also into two isomeric 3-hydroxy-5α-cholanic acids: 3α- and 3β-hydroxy-5β-cholanic acids and 3α- and 3β-hydroxy-5α-cholanic acids.[25] Furthermore, Ogura[24] reported that the particle-free fraction of rat liver homogenates showed both 3α- and 3β-hydroxysteroid dehydrogenase activity on the bile acid series. These observations lead us inevitably to the assumption that the presence of 7α-hydroxyl group provides an obligatory requirement for the stereospecific formation of 3α-hydroxylated bile acids, and probably of 5β-cholane nucleus.

We would like to propose, besides the sequence reported previously,[4] a novel route of the chenodeoxycholic acid formation from cholesterol as follows:

Cholesterol → 7α-hydroxycholesterol → 3β,7α-dihydroxychol-5-enic acid → (7α-hydroxy-3-oxochol-4-enic acid) → 7α-hydroxy-3-oxocholanic acid → chenodeoxycholic acid.

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