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In vivo Conversion of 7α-Hydroxycholesterol-^{14}C to 3β, 7α-Dihydroxychol-5-enoic-^{14}C and -4-enoic-^{14}C Acids as well as to Allocholic-^{14}C Acid in the Hen

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It was demonstrated that 7α-hydroxycholesterol-^{14}C administered to a hen furnished with a bile-fistula was converted not only to the common primary bile acids but also to 3β, 7α-dihydroxychol-5- and -4-enoic acids, the latter of which has recently been isolated from bladder bile of hens.

As a metabolite of the 7α-hydroxy sterol-^{14}C, allocholic acid was also identified and its natural occurrence in bladder bile once reported was confirmed (see "RESULTS").

In a preceding paper (*1*) we reported that 3β, 7α-dihydroxychol-4-enoic acid** was present in bladder bile of hens which might be a by-product in a new biogenetic pathway of chenodeoxycholic acid** proposed by Ayaki and Yamasaki (*2*), where 3β, 7α-dihydroxychol-5-enoic acid** was assumed to be an intermediate.

The present study was undertaken to see whether 7α-hydroxycholesterol-^{14}C, an early intermediate of bile acid biogenesis, is converted to those unsaturated (Δ^4 and Δ^5) C_{24}-bile acids in a bile-fistula hen.

MATERIALS AND METHODS

7α-Hydroxycholesterol-^{14}C–7α-Hydroxycholesterol-^{14}C was the same specimen as reported before (*3*).

3β, 7α-Dihydroxychol-5-enoic and 3β, 7α-Dihydroxychol-4-enoic Acids–3β, 7α-Dihydroxychol-5-enoic and 3β, 7α-Dihydroxychol-4-enoic acids were the same specimens as reported earlier (*1*).

Allocholic Acid–Allocholic acid was the sample obtained from Gigi-fish bile and purified (see "RESULTS").

Cholic and Chenodeoxycholic Acids–Cholic and chenodeoxycholic acids were stock samples purified in this laboratory (*1*).

Thin-layer chromatography (TLC) was performed on Kiesel gel H and G (E. Merck, Germany), and bile acids on the plates were

* The present address: Central Research Institute, TEIJIN Ltd., 1955, Hirayama, Hino-city, Tokyo. Reprints will be requested to K. Yamasaki. ** The trivial names are used: cholesterol, cholest-5-en-3β-ol; 7α-hydroxycholesterol, cholest-5-ene-3β, 7α-diol; lithocholic, chenodeoxycholic and cholic acids, 3α-monohydroxy-, 3α, 7α-dihydroxy- and 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acids; 3β, 7α-dihydroxychol-5- and -4-enoic acids, 3β, 7α-dihydroxychol-5- and -4-en-24-oic acids; allocholic acid, 3α, 7α, 12α-trihydroxy-5α-cholan-24-oic acid.

localized with phosphomolybdic acid reagent (*4*).

Radioactivity of each bile acid sample was assayed with a windowless 2π gas flow counter (Toshiba Model DEF-1501 K), μCi of ^{14}C giving a count of 9.7×10^5 cpm.

Thin-layer radioscannograms were taken with a thin-layer radiochromatogram scanner (Aloka Thin-Layer Chromatogram Scanner, Model TRM-1B).

Gas chromatography was performed with a gas chromatograph (Shimadzu, Model GC-1C): column, 0.75% SE-52 (1.5 m×4 mm); temperature 240°C.

Animal Experiments—After 24 hr fasting, an adult hen (white leghorn) weighing about 2 kg was furnished with a bile-fistula. Laparotomy was performed under intramuscular thiamylal and open drop of ether, and the right part of the keel bone was partially resected for easy access to the gall bladder.

The hen used had two bile ducts, one directly reaching the duodenum and the other by way of a gall bladder. A fistula was set to the latter duct in the usual way, the former one being completely ligated.

After 24 hr canalization of the duct, the hen was injected intraperitoneally with 1.6 mg of 7α-hydroxycholesterol-^{14}C (8.6×10^5 cpm), which was dissolved in a minimum volume of ethanol and emulsified with 20 ml of Ringer's solution containing 2 drops of Tween 20.

The fistula bile was collected during 7 days in a flask containing ethanol and filtered. About 87% of the radioactivity administered was recovered. The filtrate was concentrated and hydrolyzed in 2 N KOH at 130°C for 3 hr. The hydrolysate was diluted with water and extracted twice with ether to remove neutral lipids.

The aqueous layer was acidified with dilute HCl and extracted three times with ether. The combined ethereal extracts were washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness to give 550 mg of acidic fraction.

An aliquot of the above fraction was subjected to reversed phase column chromatography according to Norman and Sjövall (*5*),

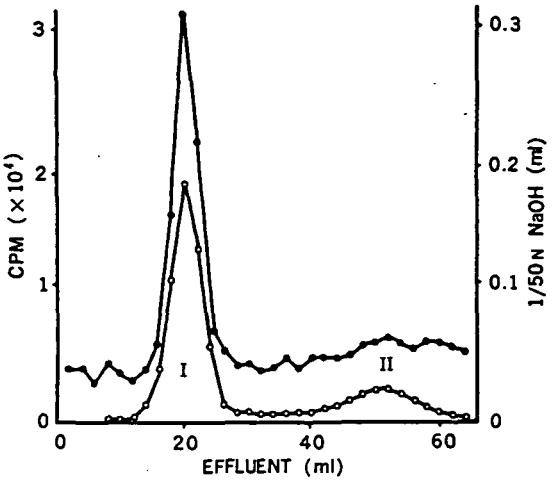

Fig. 1. Reversed phase chromatography of the acidic fraction of the hydrolyzed bile obtained from the bile-fistula hen given 7α-hydroxycholesterol-^{14}C. Column, 4.5 g of hydrophobic Celite; solvent system, F (*5*). ○, radioactivity; ●, titration values. I, cholic acid; II, chenodeoxycholic acid.

solvent system F being used. As shown in Fig. 1, the titration curve coincided fairly well with that of radioactivity, in contrast to the case of bile-fistula rat given 7α-hydroxycholesterol-^{14}C (*3*). In spite of the finding that the major bile acid of bladder bile of hens was chenodeoxycholic acid (*1*), both titration and radioactivity peaks corresponding to chenodeoxycholic acid (II) were very low compared to those of cholic acid (I). Thin-layer chromatography of another aliquot also showed a markedly large spot corresponding to cholic acid compared to that of the chenodeoxycholic acid fraction.

Analysis of the Acidic Fraction—The title fraction dissolved in chloroform : methanol (1 : 1, v/v) was loaded on several Kiesel gel plates (20×20 cm, 1 mm thick). They were developed once with isooctane : ethyl acetate : acetic acid (20 : 20 : 4, v/v) until the solvent front moved as high as about 15 cm. After being air-dried, each plate was again developed with the same solvent system until the solvent front reached the top of the plate. After being marked with iodine vapor (*6*), each plate was divided into three zones, F_1, F_2 and F_3, corresponding to cholic (*Rf* 0.14–

0.30), chenodeoxycholic (*Rf* 0.36–0.60) and lithocholic (*Rf* 0.67–0.90) acids respectively. Since the last named one had no radioactivity, it was not further investigated.

RESULTS

I. Analysis of Zone F_1—This zone of each plate was scraped off and eluted with methanol. The combined eluates were evaporated to dryness and extracted thoroughly with ether. The ethereal extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was subjected to TLC (Kiesel gel H plate) in isooctane : ethyl acetate : acetic acid (40 : 40 : 4, v/v). The plate was marked with iodine vapor (*6*) and the band corresponding to cholic acid was scraped off and eluted with methanol. The eluate was methylated (diazomethane) and rechromatographed on a Kiesel gel G plate in cyclohexane : ethyl acetate : acetic acid (7 : 23 : 3, v/v), followed by radioscannography. The plate was marked with iodine vapor as above to give three parts, 1, 2 and 3, as shown in Fig. 2a. These parts were separately eluted with ethyl acetate and rechromatographed on a Kiesel gel G plate in the same solvent system.

The results showed that parts 1 and 2 corresponded well to methyl cholate and allocholate respectively, as seen in Fig. 2b. Part 3 showed no radioactivity.

1. Identification of cholic acid: The eluate of part 1 was mixed with 50 mg of authentic methyl cholate and recrystallized to constant radioactivity. As shown in Table I, part 1 was identified as cholic acid (methyl ester).

2. Identification of allocholic acid—It is known that separation of the title acid from cholic acid by usual recrystallization is unsuccessful (*7, 8*).

As will be seen in Sec. III, methyl cholate and allocholate could be separated from one another by repeated TLC. The above purified sample (part 2 in Fig. 2a) was rechromatographed on a Kiesel gel G plate in the same solvent system mentioned above and radioscannographed. As seen in Fig. 3, the material thus obtained showed only one radioactive peak coincident well with authentic methyl allocholate.

II. Analysis of Zone F_2—This zone of each plate was scraped off, eluted with methanol and methylated with diazomethane. On TLC of an aliquot of the combined eluates in isooctane : ethyl acetate : acetic acid (40 :

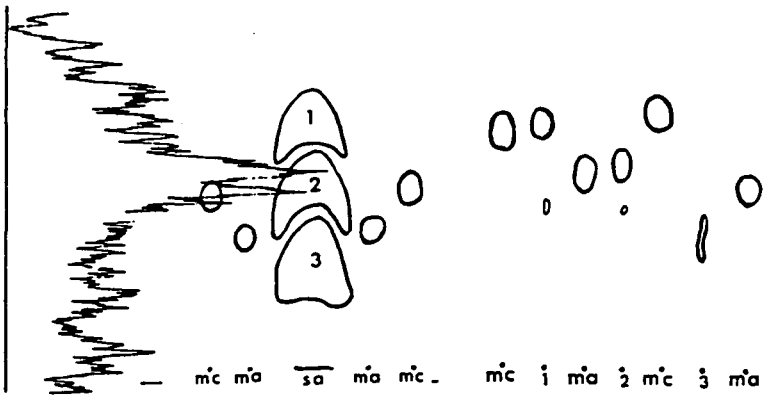

Fig. 2. a: Chromatography of zone F_1 on Kiesel gel G. Solvent system, see text. mc, methyl cholate; ma, methyl allocholate; sa, sample of zone F_1. Spots of the sample were marked with iodine vapor and radioscannographed. Reference samples were revealed with the phosphomolybdic acid reagent. b: TLC of parts 1, 2 and 3 in Fig. 2a.

TABLE I. Co-Crystallization of part 1 with methyl cholate (50 mg).

No. of recrystall.	Solvent	Weight (mg)	Specific radioactivity (cpm/mg)
Methyl ester			
1	methanol	31.4	223
2	methanol	17.2	200
3	ethyl acetate	12.3	240

20 : 4, v/v) two Lifschütz-positive spots were detected, one (LPC-I) being more polar and the other (LPC-II)* less polar than methyl chenodeoxycholate as reported earlier (*1*). LPC-I coincided well with methyl 3β,7α-dihydroxychol-5-enoate. The F_2-eluates were subjected to prepartive TLC in the same solvent system, marked with iodine vapor and the zones corresponding to methyl chenodeoxycholate (part 1) and 3β,7α-dihydroxychol-5-enoate (LPC-I) (part 2) were scraped off and separately eluted with methanol.

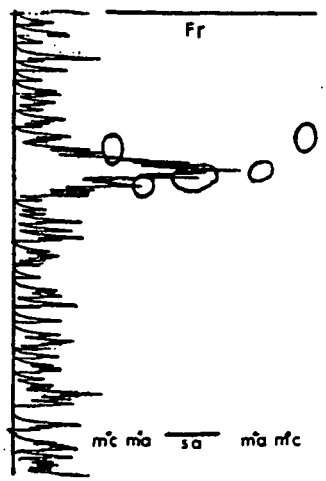

Fig. 3. Radioscannography of methyl allocholate obtained from part 2 in Fig. 2a. Remarks are the same as in Fig. 2. Fr, solvent front.

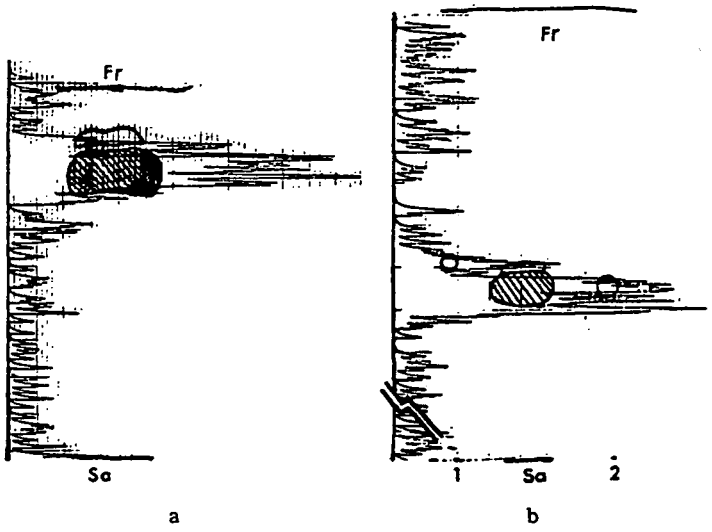

Fig. 4. a: TLC (solvent system a) and radioscannography of zone F_2 followed by iodine-vapor treatment. The hatched (iodine-stained) area (Sa) indicates the zone corresponding to LPC-I (closed area revealed with the Lifschütz reagent). Fr, solvent front. b: TLC (solvent system b) and radioscannography of the hatched area in Fig. 4a. The hatched (iodine-stained) area (Sa, LPC-I) corresponds to methyl 3β,7α-dihydroxychol-5-enoate (*2*). 1, methyl chenodeoxycholate.

* LPC-II was not further investigated.

1. Identification of LPC-I: The eluate of part 2 was submitted to TLC (Kiesel gel G) successively in two different solvent systems (a and b), isooctane : ethyl acetate : acetic acid (a, 20 : 40 : 4, v/v; b, 40 : 20 : 4, v/v), where the respective zones corresponding to LPC-1 or methyl 3β, 7α-dihydroxychol-5-enoate were marked with iodine vapor, as shown in Figs. 4a and 4b. They were scraped off from the plates and eluted with methanol. As seen in the figures, the iodine marked (hatched) areas coincided well with the radioactive peaks. The eluate of the area corresponding to LPC-I in Fig. 4b was rechromatographed on a $AgNO_3$-impergnated plate (Kiesel gel G) in isooctane : ethyl acetate : acetic acid (40 : 20 : 2, v/v) (*1*) and marked with iodine vapor. As shown in Fig. 5, LPC-I of Fig. 4b was sharply divided into two parts, the upper one corresponding to methyl 3β, 7α-dihydroxychol-5-enoate and the lower one to methyl 3β, 7α-dihydroxychol-4-enoate. The results were well accordant with those reported earlier (*1*).

These two parts were scraped off and separately eluted with chloroform. After evaporation of the solvent, the respective residues were acetylated in the usual way and the solvent was evaporated in a stream of nitrogen. Each product dissolved in a small volume of acetone was subjected to gas chromatography, acetyl methyl ester of chenodeoxycholic acid being used as an internal marker. As indicated in Table II, LPC-I was composed of methyl 3β, 7α-dihydroxychol-5- and -4-enoates.

2. Identification of chenodeoxycholic acid: The eluate corresponding to methyl chenodexycholate (part 1) was acetylated in the usual way and the reaction mixture was evaporated to dryness. To the residue was added authentic acetyl methyl ester of chenodeoxycholic acid (25.1 mg) and recrystallized to constant radioactivity. As shown in Table III, part 1 of zone F_2 was identified as chenodeoxycholic acid (methyl ester).

III. Isolation of Allocholic Acid from Hen Bile—As was reported earlier (*1*), most part of chenodeoxycholic acid was removed from the hydrolysate of the bladder bile of hens

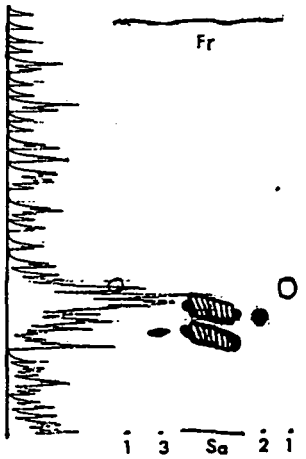

Fig. 5. TLC ($AgNO_3$-impregnated plate; solvent system b) and radioscannography of the hatched area (Sa) in Fig. 4b. 1, methyl chenodeoxycholate; 2, methyl 3β, 7α-dihydroxychol-5-enoate; 3, methyl 3β, 7α-dihydroxychol-4-enoate. The hatched areas were iodine stained. Fr, solvent front.

TABLE II. Retention times relative (RRT) to that of chenodeoxycholic acid (Ac Me ester) 1.00. Retention time of chenodeoxycholic acid (Ac Me ester) 15.6 min.

	RRT
3β, 7α-Dihydroxychol-5-enoic acid (Ac Me ester)[1]	0.34
Material of the upper part in Fig. 5	0.33
3β, 7α-Dihydroxychol-4-enoic acid (Ac Me ester)[1]	0.42
Material of the lower part in Fig. 5	0.43

[1] Ac Me ester, acetyl methyl ester.

TABLE III. Co-Crystallization of the eluate, part 1, in zone F_2 with acetyl methyl ester (Ac Me ester) of chenodeoxycholic acid (25.1 mg).

No. of recrystall.	Solvent	Weight (mg)	Specific radioactivity (cpm/mg)
Ac Me ester			
1	methanol-water	28.7	1,170
2	methanol-water	18.7	1,110
3	ether-hexane	15.1	1,110

(1.5 liters) as Ba-salt. The filtrate of the Ba-salt was acidified with dilute HCl to give the "cholic acid" fraction (11.5 g).

By repeated recrystallization from either ethyl acetate or ethanol, most part of cholic acid was removed from this fraction. The filtrates of some final recrystallizations were combined and evaporated to dryness and the residue was dissolved in hot ethyl acetate. On cooling, 600 mg of semi-crystalline material was separated as reported earlier (*1*). This was recrystallized from ethanol and another crop of fairly pure cholic acid (150 mg) was obtained. The mother liquor was evaporated to dryness and the residue was recrystallized twice from ethyl acetate to give 400 mg of crystals, mp 198–202°C. They were methylated (diazomethane) and recrystallized from ether-petroleum ether. Colorless needles (330 mg), mp 206–209°C.

The methyl ester dissolved in chloroform: methanol (1 : 1, v/v) was divided into three parts and subjected to preparative TLC (Kiesel gel G plate; 20×20 cm), a solvent system, isooctane: ethyl acetate: acetic acid (20 : 40 : 4, v/v), being used. Each plate was dried in air and again developed with the same solvent system. These procedures were further repeated twice. Each plate was marked with iodine vapor (*6*) and the area corresponding to methyl allocholate* was scraped off and eluted with ethyl acetate. The combined eluates were concentrated to give 26 mg of crystals, mp 223–224°C [reported mp 225°C (*9*)]. This material was hydrolyzed overnight in a 5% alcoholic KOH at room temperature and acidified with dilute HCl. The precipitate was recrystallized from acetone to give fine needles of allocholic acid, mp and mixed mp 239–241°C [reported mp 239–241°C (*9*)]; positive tests of Hammarsten's and Mylius' reactions (purple and red in color respectively).

Infra-red spectrum of this acid was superimposable to that reported by Anderson and Haslewood (*9*), as shown in Fig. 6.

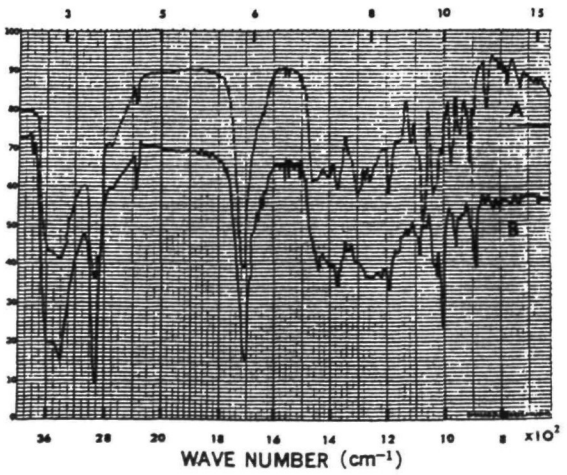

Fig. 6. Infra-red spectra in KBr. A, IR spectrum of cholic acid; B, IR spectrum of allocholic acid.

DISCUSSION

3β,7α-Dihydroxychol-5-enoic-^{14}C and 3β,7α-Dihydroxychol-4-enoic-^{14}C Acids—The present experiments showed that 7α-hydroxycholesterol-^{14}C was converted not only to the usual primary bile acids but also to the title unsaturated bile acids, one (Δ^4-acid) of which has recently been isolated from bladder bile of hens (*1*).

It must be emphasized that the title radioactive unsaturated bile acids were detected by TLC as visible spots, which might indicate that these acids are derived not only from the 7α-hydroxy sterol-^{14}C administered but also from endogenous cholesterol. Ayaki *et al.* have preliminarily reported that 3β,7α-dihydroxychol-5-enoic acid is found in fistula-bile of rat and actually derived not only from cholesterol-^{3}H administered but also from cholesterol-^{14}C formed *in situ* from mevalonate-^{14}C (*10*). These findings seem to support strongly the new pathway of chenodeoxycholic acid biogenesis proposed by Ayaki and Yamasaki (*2*).

The presence of radioactive 3β,7α-dihydroxychol-4-enoic acid in the fistula bile of hen administered with 7α-hydroxycholesterol-^{14}C suggested that 3-oxo-7α-hydroxychol-4-enoic acid is an intermediate of chenodeoxy-

* The reference compound was obtained from Gigi-fish bile (*8*) and purified by TLC.

cholic acid biogenesis (*1*), which has preliminarily been reported by Ikawa and Yamasaki (*11*).*

Allocholic Acid–It was found for the first time by one (K.Y.) of us that Ohta's acid existed also in bile of birds (hens) (*12*), which is now known to be a mixture of cholic and allocholic acids (*13*). This was here clearly confirmed by isolation of pure crystals of allocholic acid from hen bile (see "RESULTS".)*

Anderson *et al.* (*7, 9*) reported later that this acid was isolated from King-penguin bile, whereas it could not be found in chicken bile in England (*14*). Haslewood, then, suggested that this acid was derived from diet (*14*). Evidence, however, was presented in this report that this 5α-bile acid is actually a primary bile acid, because 7α-hydroxycholesterol-^{14}C was converted to this acid together with the common primary bile acids in the hen furnished with a bile fistula.

* See also the preceding paper (*1*).

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