DIFFERENCE BETWEEN CHOLIC ACID AND CHENODEOXYCHOLIC ACID IN DEPENDENCE UPON CHOLESTEROL OF HEPATIC AND PLASMATIC SOURCES AS THE PRECURSOR IN RATS

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ABSTRACT

Some difference in functional pool of cholesterol acting as the precursor of bile acids is pointed out between cholic acid and chenodeoxycholic acid. In order to elucidate this problem further, some experiments were performed with rats equilibrated with [7(n)-^{3}H, 4-^{14}C]cholesterol by subcutaneous implantation. The bile duct was cannulated in one series of experiments and ligated in another. After the operation ^{14}C-specific radioactivity of serum cholesterol fell, but reached practically a new equilibrium within three days. ^{14}C-Specific radioactivity of serum cholesterol as well as of biliary bile acids in bile-fistula rats and urinary bile acids in bile duct-ligated rats was determined during a three days-period in the new equilibrated state. The results were as follows: (1) ^{14}C-Specific radioactivity of cholic acid and chenodeoxcycholic acid in bile was lower than that of serum cholesterol, and ^{14}C-specific radioactivity of cholic acid was clearly lower than that of chenodeoxycholic acid. (2) ^{14}C-Specific radioactivity of cholic acid and β-muricholic acid in urine was lower than that of serum cholesterol, and ^{14}C-specific radioactivity of cholic acid was lower than that of β-muricholic acid. (3) Biliary as well as urinary β-muricholic acid lost tritium label at 7-position entirely during the course of formation from [7(n)-^{3}H, 4-^{14}C]cholesterol.

INTRODUCTION

A previous report of ours (1) has shown that in perfusion experiments with isolated rat livers cholesterol synthesized newly from [1-^{14}C]acetate *in situ* was incorporated into both cholic acid and chenodeoxycholic acid in a similar way, while infused lipoprotein-[1,2-^{3}H]cholesterol was incorporated into chenodeoxycholic acid at a

higher rate.

In the present work, for further confirmation of the findings, some experiments were performed with rats equilibrated with radioactive cholesterol by subcutaneous implantation.

MATERIALS AND METHODS

Materials —— Cholesterol and cholic acid were obtained from commercial sources, recrystallized three times from appropriate solvents and proved to be pure by various criteria. Chenodeoxycholic acid, α-muricholic acid, and β-muricholic acid were the same specimens described previously (1). [7(n)-^{3}H]-Cholesterol (8 Ci/mmol) and [4-^{14}C]cholesterol (58.4 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, England). These were purified by column chromatography on silicic acid (AR, 100 mesh, Mallinckrodt Chemical Works, St. Louis, U.S.A.), eluted with various n-hexane/chloroform mixtures (2). Their radiochemical purity was examined by radioscannography after development on a thin-layer plate coated with silica gel G (E. Merck AG., Darmstadt, Germany) in cyclohexane/ethyl acetate (3 : 2, v/v) and proved to be more than 99%. According to the technical data from the Radiochemical Centre, tritium in [7(n)-^{3}H]cholesterol was located at 7α (42.5%), 7β (49.5%), and 4β (8%) positions. The purified specimens of [7(n)-^{3}H]cholesterol and [4-^{14}C]cholesterol were mixed and cocrystallized three times with unlabeled material to afford crystals of [7(n)-^{3}H, 4-^{14}C]cholesterol (specific activity: ^{3}H, 1.03 x 10^{7} dpm/mg; ^{14}C, 6.19 x 10^{5} dpm/mg; ^{3}H/^{14}C ratio, 16.67).

Animals and their treatment —— Male rats of the Wistar strain, weighing 190-210 g, were anesthetized with ether, and gelatin capsules containing 81-89 mg of [4-^{14}C] or [7(n)-^{3}H, 4-^{14}C]cholesterol were implanted subcutaneously in the dorsal region according to Wilson (3). Throughout the experiment, the rats were kept in a room maintained at 23°C under a controlled lighting schedule (light on at 6:00 a.m. and off at 6:00 p.m.). They were fed on a commercial rat diet (Clea CE-2, Nihon Clea Co., Tokyo, Japan) and water ad libitum. Blood samples were obtained into a small heparinized glass tube by cutting the end of the tail. Serum was separated by centrifuging clotted blood. Three weeks after implantation the rats were used for the following experiments.

In a series of experiments, the bile duct was cannulated with a polyethylene tubing No. 10 (Igarashi Ika Kogyo Co., Tokyo, Japan) under light anesthesia with ether at about 10 a.m. The cannulated animals were kept individually in restricted cages with free access to food and 0.9% sodium chloride solution. Bile was collected at 24-h intervals for 7 days after cannulation.

In another series of experiments, the common bile duct was doubly ligated above the entry of pancreatic ducts and was cut between the ligatures. The operation was made at about 10 a.m. The bile duct-ligated rats were kept in an individual metabolic cage. Urine was collected in 24-h fractions during 7 days after ligation.

Analysis of cholesterol —— Serum total cholesterol was determined enzymatically with Determiner® TC "5" (Kyowa Medex Co., Ltd., Tokyo, Japan)

For assay of radioactivity in serum cholesterol, 80 µl of serum was mixed with 2.4 ml of cholesterol solution (10.0 mg/dl) in ethanol/acetone (1 : 1, v/v). The mixture was saponified with two drops of 33% potassium hydroxide solution at 45°C for 30 min and neutralized with 10% acetic acid by making use of 0.1% phenolphthalein solution as an indicator. One ml of 0.5% ethanolic solution of digitonin was added and the mixture was allowed to stand for 1 h. Cholesterol digitonide was precipitated by centrifugation, washed with ether, dried at 90°C for 30 min, dissolved in methanol and radioassayed.

Analysis of bile acids —— Analytical procedure of bile acids was as described before (1). Briefly, bile was extracted with hot ethanol and the filtrate was evaporated to dryness. The residue was partitioned between ethnaol/water and n-heptane/ether (1 : 1 : 1 : 1, by vol.). The aqueous ethanol layer was evaporated to dryness and the residue was hydrolyzed in alkaline solution. Bile acids were extracted with ether from the hydrolyzate after acidification with dilute hydrochloric acid. Bile acids were separated into four fractions by column chromatography on silica gel, eluted with various chloroform/methanol mixtures. Bile acids in each fraction were methylated and the methyl esters were isolated by preparative thin-layer chromatography on plates of silica gel H, and passed through a column of Sephadex-LH 20. They were acetylated with acetic anhydride. The resulting methyl esters of acetylated bile acids were quantitated by gas chromatography on a column of 3% OV-1. Their radioactivity was determined in a liquid scintillation spectrometer.

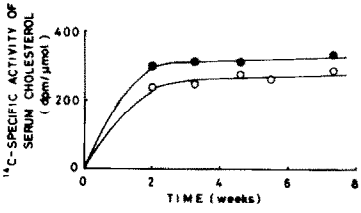
Urine was applied to an Amberlite XAD-2 column and bile acids were eluted with methanol according to the method of Makino and Sjövall (4). After hydrolysis, they were analyzed as described above.

Oxidation of cholic acid to dehydrocholic acid —— Doubly labeled cholic acid which was isolated as a metabolite of [7(n)-^{3}H, 4-^{14}C]cholesterol was mixed with standard specimen (methyl ester derivative). One part of the mixture dissolved in 50 parts of acetic acid [w/v(g/ml)] was treated with 6 parts [v(ml)] of 20% chromic anhydride-acetic acid solution with cooling. The reaction mixture was allowed to stand for about 10 min at room temperature and about 10 volumes of water was added gradually. Semicrystalline precipitate formed was collected by filtration after two hours' standing and washed with water. Recrystallization of this material was repeated from acetic acid/water or methanol/water to attain a constant ^{3}H/^{14}C ratio. Sufficient purity was confirmed by melting point and mixed melting point [reported mp 232 - 233°C (5)] and thin-layer chromatography on a plate of silica gel H in benzene/acetone (3 : 2, v/v).

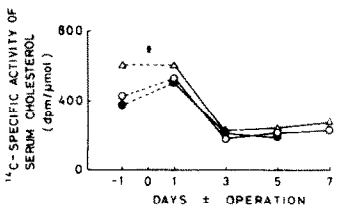
RESULTS

Specific radioactivity of serum cholesterol after implantation of [4-^{14}C]cholesterol —— Fig. 1 illustrates specific radioactivity

curves of serum cholesterol after subcutaneous implantation of capsules containing [4-^{14}C]cholesterol in preliminary experiments. Two weeks after implantation specific radioactivity attained practically a plateau. The data confirmed those of Wilson (3).



(Fig. 1)



(Fig. 2)

Fig. 1. The change in specific radioactivity of serum cholesterol after subcutaneous implantation of capsules containing [4-^{14}C]cholesterol in preliminary experiments. Open circle: 80.2 mg (0.09 μCi/mg), closed circle: 82.6 mg (0.09 μCi/mg).

Fig. 2. The change in ^{14}C-specific radioactivity of serum cholesterol after bile duct-cannulation in rats equilibrated with [7(n)-^{3}H, 4-^{14}C]cholesterol. Three weeks after implantation of the doubly labeled cholesterol [open circle: 80.5 mg, closed circle: 86.4 mg, open triangle: 85.2 mg (4.68 μCi of ^{3}H and 0.28 μCi of ^{14}C per mg)] the bile duct was cannulated (indicated by an arrow).

Experiments in bile-fistula rats —— Three weeks after implantation of capsules containing [7(n)-^{3}H, 4-^{14}C]cholesterol, the bile duct was cannulated and bile was collected in 24-h fractions for 7 days. The change in ^{14}C-specific radioactivity of serum cholesterol is shown in Fig. 2. The specific radioactivity decreased remarkably during 3 days after cannulation and remained almost constant thereafter.

Bile samples on day 4,5, and 6 were analyzed for bile acids. The change in ^{14}C-specific radioactivity of bile acids relative to that of serum cholesterol are shown in Table 1. The relative specif-

ic radioactivity of bile acids generally tended to decrease during 3 days. The relative specific radioactivity of cholic acid was always lower than that of chenodeoxycholic acid. The relative specific radioactivity of both α-muricholic acid and β-muricholic acid was rather closer to that of chenodeoxycholic acid than to that of cholic acid.

Table I. The ^{14}C-specific radioactivity of bile acids relative to that of serum cholesterol after bile duct-cannulation in rats equilibrated with [7(n)-^{3}H, 4-^{14}C]cholesterol. Bile samples were collected in 24-h fractions during 7 days after cannulation. Bile acids were isolated from hydrolyzed bile samples on day 4,5, and 6 by thin-layer chromatography, and their ^{14}C-specific radioactivity was determined by both radioassay and gas chromatography. Values are expressed as ^{14}C-specific radioactivity relative to that of serum cholesterol. The values of serum cholesterol here used are the means of three values for day 4,5, and 6 after bile duct-cannulation. Results are expressed as means (± S.E.) of 3 or 2 observations.

Bile acid	Relative ^{14}C-specific radioactivity		
	Days after cannulation		
	4	5	6
Cholic acid	0.74 ± 0.04	0.63 ± 0.02	0.64 ± 0.06[a]
Chenodeoxycholic acid	0.88 ± 0.05	0.76 ± 0.01	0.69 ± 0.05[a]
α-Muricholic acid	0.83 ± 0.08	0.83 ± 0.06[a]	0.74 ± 0.07[a]
β-Muricholic acid	0.81 ± 0.08	0.72 ± 0.04	0.67 ± 0.05[a]

[a]Two observations.

The tritium label of [7(n)-^{3}H, 4-^{14}C]cholesterol was partially lost during the conversion to bile acids (Table II). Retention of tritium in bile acids was 54 to 60% for cholic acid, chenodeoxycholic acid, and α-muricholic acid, while only 10% for β-muricholic acid.

Some of the isolated cholic acid was pooled, mixed with an appropriate amount of standard specimen, and oxidized with 20% chromic acid solution to dehydrocholic acid. This acid retained 8.8% of the

tritium label of precursor [7(n)-^{3}H, 4-^{14}C]cholesterol.

Table II. Retention of tritium in biliary bile acids formed from [7(n)-^{3}H, 4-^{14}C]cholesterol. Bile was collected in 24-h fractions during day 4 to 6 after bile duct-cannulation in rats equilibrated with [7(n)-^{3}H, 4-^{14}C]cholesterol. Bile acids were isolated chromatographically and radioassayed for determination of the ^{3}H/^{14}C ratio. Retention of tritium in bile acids is given by per cent of ^{3}H-radioactivity of that in precursor [7(n)-^{3}H, 4-^{14}C]cholesterol. Results are expressed as means (± S.E.) of 3 or 2 observations.

Bile acid	^{3}H-Retention in bile acids (%)		
	Days after cannulation		
	4	5	6
Cholic acid	58.5 ± 0.97	56.6 ± 0.40	56.5 ± 0.10[a]
Chenodeoxycholic acid	54.5 ± 0.28	54.7 ± 0.63	54.5 ± 0.45[a]
α-Muricholic acid	60.9 ± 0.43	54.2 ± 2.10[a]	54.4 ± 0.15[a]
β-Muricholic acid	10.7 ± 0.23	10.0 ± 0.14	9.7 ± 0.24[a]

[a]Two observations.

Experiments in bile duct-ligated rats —— Similar experiments were carried out with bile duct-ligated rats equilibrated with [7(n)-^{3}H, 4-^{14}C]cholesterol. After ligation of the bile duct, ^{14}C-specific radioactivity of serum cholesterol decreased gradually during 3 days and showed no remarkable fluctuation thereafter (Fig. 3).

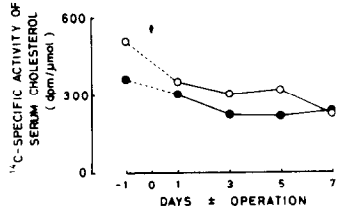


Fig. 3. The change in ^{14}C-specific radioactivity of serum cholesterol after bile duct-ligation in rats equilibrated with [7(n)-^{3}H, 4-^{14}C]cholesterol. Three weeks after implantation of the doubly labeled cholesterol [open circle: 85.9 mg, closed circle: 81.4 mg (the same specific radioactivity as described in Fig. 2)] the bile duct was ligated (indicated by an arrow).

Urine samples collected on day 4,5, and 6 after bile duct-ligation were analyzed for bile acids. Table III shows ^{14}C-specific radioactivity of cholic acid and β-muricholic acid relative to that of serum cholesterol. The relative ^{14}C-specific radioactivity of β-muricholic acid was obviously higher than that of cholic acid.

Table III. ^{14}C-Specific radioactivity of urinary bile acids relative to that of serum cholesterol after bile duct-ligation in rats equilibrated with [7(n)-^{3}H, 4-^{14}C]cholesterol. Urine samples were collected in 24-h fractions during day 4 to 6 after bile duct-ligation. Bile acids were isolated chromatographically and their ^{14}C-specific radioactivity was determined by both radioassay and gas chromatography. Values are expressed as ^{14}C-specific radioactivity relative to that of serum cholesterol. The values of serum cholesterol here used are the means of three values for day 4,5, and 6 after bile duct-ligation. Results are expressed as means of a pair of rats.

Bile acid	Relative ^{14}C-specific radioactivity		
	Days after ligation		
	4	5	6
Cholic acid	0.65 ± 0.11	0.54 ± 0.09	0.74 ± 0.07
β-Muricholic acid	0.72 ± 0.08	0.68 ± 0.11	0.77 ± 0.09

Retention of tritium in the isolated cholic acid and β-muricholic acid are shown in Table IV. The retention was 32 to 41% for cholic acid, while 8% for β-muricholic acid. Thus, urinary cholic acid in this experiment showed a lower rate of tritium retention when compared with biliary cholic acid in the experiment with bile-fistula rats (cf. Table II). When urinary cholic acid was oxidized with chromic acid to dehydrocholic acid, retained tritium label was 8.2%.

It was difficult to obtain reliable data with chenodeoxcyholic acid and α-muricholic acid owing to their scanty mass and radioactivity.

Table IV. Retention of tritium in urinary bile acids formed from [7(n)-^{3}H, 4-^{14}C]cholesterol. Urine was collected in 24-h fractions during day 4 to 6 after bile duct-ligation in rats equilibrated with [7(n)-^{3}H, 4-^{14}C]cholesterol. Bile acids isolated chromatographically were radioassayed for determination of the ^{3}H/^{14}C ratio. Rentention of tritium in bile acids is given by per cent of ^{3}H-radioactivity of that in precursor [7(n)-^{3}H, 4-^{14}C]cholesterol. Results are expressed as means of a pair of rats.

Bile acid	^{3}H-Retention in bile acids (%)		
	Days after ligation		
	4	5	6
Cholic acid	32.9 ± 10.2	36.0 ± 8.3	41.3 ± 5.5
β-Muricholic acid	8.2 ± 0.3	8.0 ± 0.1	8.0 ± 0.1

DISCUSSION

When rats were implanted subcutaneously with a capsule containing radioactive cholesterol, specific radioactivity of serum cholesterol reached a plateau in approximately 3 weeks in accordance with the findings of Wilson (3). When the bile duct of rats equilibrated with radioisotopic cholesterol under such a condition was cannulated or ligated, specific radioactivity of serum cholesterol decreased during 3 days after operation and became nearly constant thereafter. A possible explanation for this observation is that radioactive cholesterol in serum was diluted with cholesterol synthesized at a rate higher than that in the normal state and a new steady state was realized, since bile duct-cannulation accelerates the synthesis of hepatic cholesterol (6,7), and biliary obstruction also does so and increases the concentration of serum cholesterol (8-10).

^{14}C-Specific radioactivity of bile acids formed during this experimental period was generally lower than that of serum cholesterol.

This means that bile acids are formed from compartmentalized functional pool(s) of cholesterol that equilibrates rapidly with newly synthesized cholesterol in liver. The findings are consistent with those previously reported (1,11).

In bile-fistula rats, relative ^{14}C-specific radioactivities of cholic acid and chenodeoxycholic acid were not identical for each other, i. e. specific radioactivity of chenodeoxycholic acid was always higher than that of cholic acid. This observation also supports our previous suggestion (1) that there may be a mechanism which involves cholesterol of plasmatic source as a more preferred precursor in the biosynthesis of chenodeoxycholic acid. Relative ^{14}C-specific radioactivity of α-murihcolic acid and β-muricholic acid was rather close to that of chenodeoxycholic acid. The results were indicative of the precursor-product relationship between chenodeoxycholic acid and both muricholic acids.

In bile duct-ligated rats, relative ^{14}C-specific radioactivity of urinary β-muricholic acid was higher than that of urinary cholic acid. This may be explained as follows: β-muricholic acid is derived from chenodeoxycholic acid which depends upon cholesterol of plasmatic source as the precursor more than cholic acid does.

According to the established mechanism on the formation of primary bile acids from cholesterol in the rat (12), each of cholic acid, chenodeoxycholic acid, and α-muricholic acid should retain tritium at 7β-position when derived from [7(n)-^{3}H, 4-^{14}C]cholesterol. Actually, our results with bile-fistula rats suggest that the tritium at 7β-position was retained in the isolated cholic acid, chenodeoxycholic

acid, and α-muricholic acid (Table II). Biliary cholic acid in bile-fistula rats given [7(n)-^{3}H, 4-^{14}C]cholesterol showed the theoretical retention of tritium (Table II), while urinary cholic acid in bile duct-ligated rats which received the labeled cholesterol had a somewhat lower value of retention (Table IV). The mechanism of the partial elimination of tritium in urinary cholic acid is not clear at present.

If β-muricholic acid is derived from [7β-^{3}H]α-muricholic acid *via* a 7-keto derivative, the tritium at 7β-position of α-muricholic acid will be eliminated during the conversion. In an alternative pathway, β-muricholic acid may be formed from 3α,6β-dihydroxy-5β-cholan-24-oic acid by direct hydroxylation at 7β-position (13,14). In such a case, tritium at 7α-position of the dihydroxy acid, if located, will be retained in β-muricholic acid formed. The results (Tables II and IV) implies that β-muricholic acid formed from [7(n)-^{3}H, 4-^{14}C]cholesterol retained no tritium at 7-position in either case of the *in vivo* experiments. The findings indicate that β-muricholic acid was derived from α-muricholic acid through its 7-keto derivative (15).

In our previous studies *in vivo* and/or *in vitro* (1), radioisotopic precursors of cholesterol or bile acids, *i. e.* [1-^{14}C]acetate or [1,2-^{3}H]cholesterol carried on lipoprotein showed a time lag in incorporating into α- and β-muricholic acids, especially into the latter, as compared with incorporating into chenodeoxycholic acid. Also in our studies with isolated perfused rat-livers (16), specific radioactivity of α- and β-muricholic acids was lower than that of

chenodeoxycholic acid after infusion of [24-^{14}C]chenodeoxycholic acid. The present results have failed to give a direct account of these observations.

Gréen and Samuelsson (17) have demonstrated that the major part of the tritium of [4β-^{3}H]cholesterol was eliminated but a significant part was transferred to the 6β-position during its conversion to bile acids. In the present experiments, when cholic acid biosynthesized from [7(n)-^{3}H, 4-^{14}C]cholesterol was oxidized to dehydrocholic acid, this acid retained tritium label in the same level as β-muricholic acid formed from the doubly labeled cholesterol both in bile-fistula rats and in bile duct-ligated rats. These facts suggest that the tritium label at 4-position of [7(n)-^{3}H]cholesterol preparation used in the experiments is α-oriented.

In conclusion, the findings confirm our previous suggestion that bile acids originate from a hepatic functional pool of cholesterol equilibrating rather quickly with newly synthesized cholesterol in situ, and that chenodeoxycholic acid depends upon cholesterol of plasmatic source as the precursor more than cholic acid does. Furthermore, β-muricholic acid seems to arise in vivo, starting practically from chenodeoxycholic acid, by oxido-reduction of the 7α-hydroxyl group through a 7-keto derivative, but not by direct 7β-hydroxylation of possible intermediate 3α,6β-dihydroxy-5β-cholan-24-oic acid.

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Trivial names used: cholesterol, 5-cholesten-3β-ol; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; cholic acid, 3α, 7α,12α-trihydroxy-5β-cholan-24-oic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholan-24-oic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid; dehydrocholic acid, 3,7,12-trioxo-5β-cholan-24-oic acid.