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ALTERNATIVE BIOGENETIC PATHWAYS OF C_{24}-BILE ACIDS WITH SPECIAL REFERENCE TO CHENODEOXYCHOLIC ACID

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INTRODUCTION

In most higher animals including birds and bony fish cholic acid and chenodeoxycholic acid are the main biliary metabolites (the primary bile acids) of cholesterol. The quantitative ratio of these acids in individual bladder biles is not only different from species to species, but also strongly modified by the presence of the secondary bile acids, *e. g.*, deoxycholic acid.[1] Wieland and Reverey (1924)[2] for the first time isolated chenodeoxycholic acid* (Anthropodesoxycholsäure) from human bladder bile and reported that its content therein was not less than that of deoxycholic acid, the latter acid and cholic acid being found in the ratio 1 : 3.**

Nagaki (1942)[3] and independently the author*** (1945/49)[4] demonstrated that, contrary to the German authors, most samples of human fistula bile as well as bile samples obtained by permanent catheterization**** contained chenodeoxycholic acid at nearly the same level as that of cholic acid, while deoxycholic acid was scarcely found. Such apparently contradictory findings have not been explained, until deoxycholic acid was proved to be a secondary bile acid derived from cholic acid during its entero-hepatic circulation (1959).[5]

Nevertheless it must be emphasized that chenodeoxycholic acid, especially in fistula bile, is present at nearly the same level as that of cholic acid in human bile, while in most mammals this dihydroxy bile acid was found at

* At the same time Windaus *et al.* isolated this bile acid from bladder bile of geese (Windaus, A., Bohne, A. and Schwarzkopf, E.: Z. Physiol. Chem. **140,** 23, 1924).

** Yoshioka recently reported that the ratio of cholic, chenodeoxycholic and deoxycholic acids in bladder bile of normal adults in the north-west district of Japan is 0.68 : 1 : 0.53 (Yoshioka, D.: J. Yonago Med. Ass. **29,** 1978, in Japanese).

*** Owing to the bad conditions during the World War II, Dr. Nagaki and the author, who stayed in Mantsuria (the Harbin Medical School), could not correspond with one another.

**** A number of typhoid-bacillus carriers were treated at the time by means of permanent catheterization of bile with some benign result.

Abbreviations used: cholic and allocholic acids, 3α, 7α, 12α-trihydroxy-5β- and 5α-cholan-24-oic acids; α- and β-muricholic acids, 3α, 6β, 7α- and 3α, 6β, 7β-trihydroxy-5β-cholan-24-oic acids; chenodeoxycholic and allochenodeoxycholic acids, 3α, 7α-dihydroxy-5β- and 5α-cholan-24-oic acids; ursodeoxycholic acid, 3α, 7β-dihydroxy-5β-cholan-24-oic acid; deoxycholic acid, 3α, 12α-dihydroxy-5β-cholan-24-oic acid; lithocholic and allolithocholic acids, 3α-hydroxy-5β- and 5α-cholan-24-oic acids; 5α-cyprinol, 5α-cholestane-3α, 7α, 12α, 36, 37-pentol; cholesterol, cholest-5-en-3β-ol; 7α- and 7β-hydroxycholesterols, cholest-5-ene-3β, 7α- and 3β, 7β-diols; mevalonic acid, 3, 5-dihydroxy-3-methylpentan-1-oic acid; NAD, NADP, NADH and NADPH, nicotinamide adenine dinucleotide, its phosphate and their respective reduced forms; TLC, thin layer chromatography; GLC, gas liquid chromatography; NMR, nucleomagnetic resonance.

much lower level than that of cholic acid. On the hygienical or clinical standpoint of the control of blood cholesterol level, therefore, the biogenetic study of chenodeoxycholic acid seems as much important as that of cholic acid not only biochemically but also clinically.

Most parts of the biogenetic sequence of cholic acid have recently been clarified,[6] though most enzymatic studies of the individual metabolic steps remain to be conducted. As for the biogenetic pathway of chenodeoxycholic acid, it has been assumed, at present, to be, in principle, analogous to the pathway of cholic acid biogenesis,[7] 3α, 7α-dihydroxy-5β-cholestane being a key intermediate.

However, alternative pathways of chenodeoxycholic acid biogenesis have lately been proposed by Myant's group[8] and the author's group,[9] respectively (Chart A). According to the former group, the side-chain cleavage of cholesterol molecule precedes all of the transformations of its nuclear part, 3β-hydroxychol-5-enoic acid* (B-1) being assumed as a key intermediate (pathway

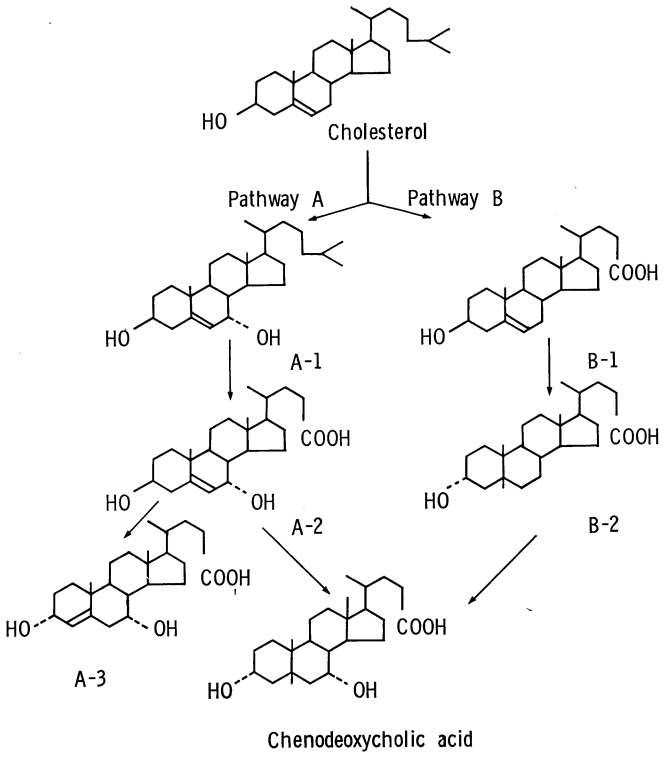

Chart A Alternative biogenetic pathways of chenodeoxycholic acid.

B). On the other hand, the author's group has proposed that 3β, 7α-dihydroxychol-5-enoic acid* (Δ^5-acid, A-2), which is the side-chain cleaved product of 7α-hydroxycholesterol (A-1), is a key intermediate to chenodeoxycholic acid (pathway A). The latter pathway has been substantiated not only by experiments *in vitro* as well as *in vivo*, but also by the isolation of the Δ^5-acid and its metabolic by-product, 3α, 7α-dihydroxychol-4-enoic acid (Δ^4-acid, A-3) from bile samples of hens and humans.

The present paper deals with a review of the data concerned with the pathway (pathway A) proposed by the author's group. At the same time the data concerned with Myant's pathway (pathway B) are described briefly and critically.

A. BIOGENESIS OF CHENODEOXYCHOLIC ACID *VIA* 3β, 7α-DIHYDROXYCHOL-5-ENOIC ACID (Δ^5-Acid)

I. *Experiments in vitro*

As will be described in II. a, when 7α-hydroxycholesterol-^{14}C was administered to a rat with a bile fistula, it was found that radioactivity of the chenodeoxycholic acid fraction relative to that of the cholic acid fraction was much higher than that of the corresponding fraction observed when cholesterol-^{14}C was given. Furthermore when the same ^{14}C-dihydroxy sterol was given to bile-fistula rats, radioactivity was taken up by the mitochondrial fraction much more than by the microsomal fraction of the liver homogenate, while a reverse distribution of radioactivity was observed when cholesterol-^{14}-C was given (Table 1).

TABLE 1. Distribution of ^{14}C in the Subcellular Fractions of the Fistula-Rat Liver (3 hr after).

Fraction	Administration of			
	7α-Hydroxycholesterol-^{14}C		Cholesterol-^{14}C	
	cpm/g, liver	%	cpm/g, liver	%
Nuclear	82	10	536	8
Mitochondrial	433	53	2,167	32
Microsomal	132	16	3,650	54
Supernatant	177	21	416	6
(Total)	(824)	(100)	(6,770)	(100)
^{14}C in the Liver	7×10^3 cpm	1.3%	44×10^3 cpm	5.5%
^{14}C in the Bile	9,7150 cpm	18.0%	4,550 cpm	0.6%

* For convenience, the systematic names of the C_{24}-bile acids used in this review are expressed in such a manner as described above, instead of 3β-hydroxychol-5-en-24-oic acid and 3β, 7α-dihydroxychol-5-en-24-oic acid.

These findings suggest that 7α-hydroxycholesterol exogenously administered is preferentially taken up by the mitochondria of liver cells, where its side-chain is cleaved to give the Δ^5-acid, which, in turn, is convered to chenodeoxycholic acid and its further oxidized product, α-muricholic acid, as will be described below (II. c).

I. a. *Metabolism of 7α-Hydroxycholesterol-^{14}C to Some C_{24}-Bile Acids*

On such a suggestion as above, the following incubation experiments were carried out, using the mitochondrial fractions prepared from the liver homogenates of rats and rabbits.

I. a. i. *With the mitochondrial fraction of rat liver.*[10]—7α-Hydroxycholesterol-^{14}C was solubilized with aqueous albumin solution and incubated for 5 hr at 37°C in air with the mitochondrial fraction in a medium shown in Table 2. The incubation mixture was extracted with chloroform and methanol.

TABLE 2. Incubation Medium

7α-Hydroxycholesterol-^{14}C	0.51μmol, 1×10^5 cpm
Glutathione	100 μmol
ATP	144 〃
NAD	94 〃
Sodium citrate	100 〃
$MgCl_2$	98 〃
Tris-HCl buffer (ph 8.4)	1.2〃
Boiled juice*	1.2 ml
Total volume	15.0 ml

* The supernatant fraction boiled

After removal of the organic solvents, the residual aqueous solution was hydrolyzed as usual (2N KOH; 130°C; 3 hr) and neutral lipids were removed. The hydrolyzate was acidified and extracted with ether to obtain the acidic metabolites. On the incubation, 7-8% of the total radioactivity incubated was recovered in the acidic fraction.

The acidic fraction was subjected portion-wise to reversed phase column chromatography on a hydropholic Celite column using solvent system F.* Hereby the following bile acids (1 mg each) were used as carriers: cholic or α-muricholic acid as a trihydroxy bile acid; chenodeoxycholic acid or the Δ^5-acid as a dihydroxy bile acid.

The chromatogram obtained with one part of the acidic fraction is shown in Fig. 1. The major radioactive peaks I, II and III coincide fairly with the

* Solvent system used for reversed phase column chromatography as follows: F or FI (Norman, A.: Acta Chem. Scand. **7**, 1413, 1953; Norman, A. and Sjövall, J.: J. Biol. Chem. **233**, 872, 1958)

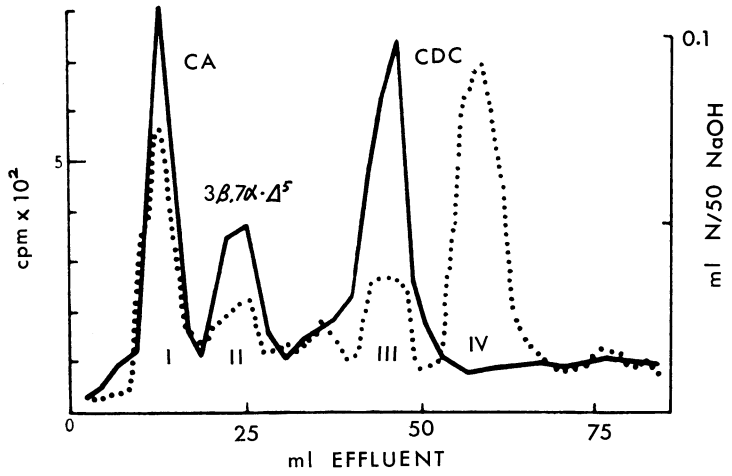

Fig. 1. Reversed phase column chromatography of the acidic fraction, with cholic acid (CA), the Δ^5-acid ($3\beta,7\alpha$-Δ^5) and chenodeoxycholic acid (CDC) added as references. Broken line, radioactivity; solid line, titration values.

respective titration peaks of the cholic acid, the Δ^5-acid and chenodeoxycholic acid added as carriers. Aliquots of the effluents were checked by TLC to collect the trihydroxy bile acid zone and the dihydroxy bile acid zone of the acidic fraction.

Except for cholic acid, such bile acids as α-muricholic acid, the Δ^5-acid and chenodeoxycholic acid were identified as such by isotope dilution technique. Although β-muricholic acid was possibly expected as a metabolite, it could not be found in the acidic fraction.

It should be noted that a prominent peak of radioactivity, peak IV, was observed in a less polar region than that of chenodeoxycholic acid, as shown in Fig. 1. As will be discussed later (IV. a. ii), this peak proved to correspond to an artifact (a methoxy-compound) derived from the newly formed Δ^5-acid, which has an allyl alcohol group in the molecule.

I. a. ii. *With the mitochondrial fraction of rabbit liver*[11]—7α-Hydroxy-cholesterol-^{14}C was incubated with the mitochondrial fraction prepared from rabbit liver homogenate in the similar manner to that described above (I. a. i). The extract obtained from the incubation mixture was hydrolyzed and, after removal of neutral lipids, the hydrolyzate was acidified with dilute HCl and extracted with ether together with chenodeoxycholic acid and the Δ^5-acid as carriers. The extract was subjected to reversed phase column chromatography as above, solvent system FI being used.

As shown in Fig. 2, three radioactive peaks I, II and III were well coincident with the three titration peaks. The metabolites corresponding to radioactive peaks I and II were identified by isotope dilution technique as the Δ^5-acid and chenodeoxycholic acid, respectively.

As will be discussed below (IV. a. ii), radioactive peak III proved to correspond to an artifact (a methoxy-compound) derived from the newly formed Δ^5-acid, as was the case with peak IV (I. a. i).

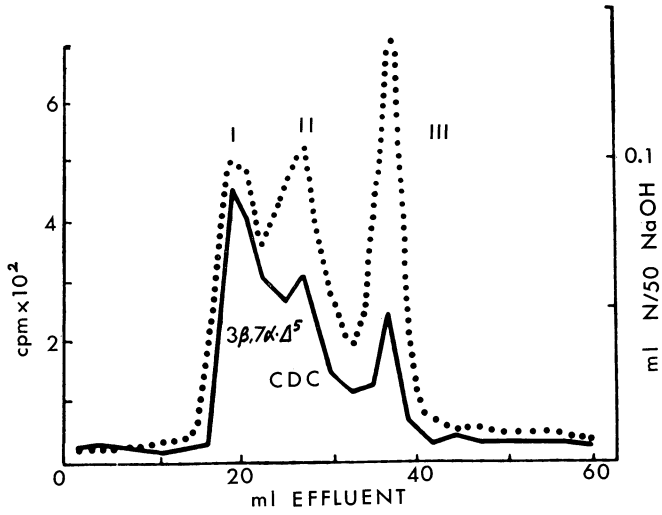

Fig. 2. Reversed phase column chromatography of the acidic fraction, with the Δ^5-acid ($3\beta,7\alpha$-Δ^5) and chenodeoxycholic acid (CDC) added as references. Broken line, radioactivity; solid line, titration values.

I. b. *Metabolism of the Δ^5-Acid with Rat Liver Homogenate*

As will be described in the next chapter (II. c), the Δ^5-acid is effectively metabolized to chenodeoxycholic acid in experiments *in vivo* with rats.

I. b. i. *Metabolism of the-Δ^5acid*—Ikawa[12] carried out incubation experiments of the Δ^5-acid, using the subcellular fractions, especially the microsomal fraction, of rat liver homogenate to get more precise information on this problem.

TABLE 3. Incubation Medium

$3\beta,7\alpha$-Dihydroxychol-5-enoic-^{14}C acid	0.5 μmol
0.2M Tris-HCl buffer (pH 9.0)	0.4 ml
0.15M Nicotinamide	0.4 〃
The microsomal fraction	2.0 〃
NADP	4.4 μmol
Total volume	4.0 ml

The ^{14}C-labeled Δ^5-acid was incubated under the condition indicated in Table 3. The extract of the incubation mixture was subjected to reversed phase column chromatography (solvent system F). As shown in Fig. 3, two radioactive peaks I and II were observed, where the titration curves of cholic acid and chenodeoxycholic acid were simultaneously depicted.

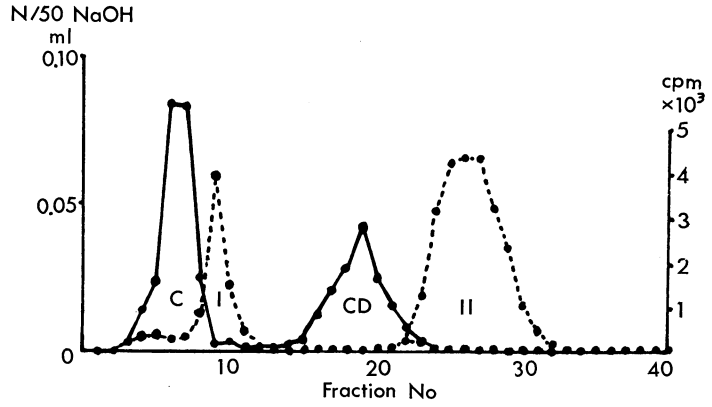

Fig. 3. Reversed phase column chromatography of the metabolites of the Δ^5-acid-^{14}C, together with cholic (C) and chenodeoxycholic (CD) acids. Solid line, titration values; broken line, radioactivity.

Peak I was proved to correspond to the starting material unchanged. Peak II corresponded neither to chenodeoxycholic acid nor to the expected metabolite, 7α-hydroxy-3-oxochol-4-enoic acid. But the metabolite corresponding to peak II was converted to the latter acid by treating with dilute HCl at room temperature. This finding indicates that the metabolite concerned is 7α-hydroxy-3-oxochol-5-enoic acid and that a 3β-hydroxysteroid dehydrogenase activity is present in the microsomal fraction of rat liver.

Further informations on this enzymatic activity were reported. (i) This activity was also present in the mitochondrial fraction, but not in the supernatant fraction of rat liver homogenate; (ii) this activity required NADP as a preferential cofactor; optimal pH round 9; (iii) the microsomal fraction catalyzed the reverse reaction of dehydrogenation of the Δ^5-acid; (iv) this activity was scarcely influenced by biliary drainage, but lost almost completely by sonication.

I. b. ii. *Metabolism of 7α-hydroxy-3-oxochol-4-enoic acid*[13]—7α-Hydroxy-3-oxochol-4-enoic-^{14}C acid was incubated in air at 37°C for 2 hr with the subcellular fractions obtained from the liver of fasting rat. The incubation medium is shown in Table 4.

TABLE 4. Incubation Medium

7α-Hydroxy-3-oxochol-4-enoic acid	1 μmol
0.15M Nicotinamide	1.0 ml
0.2M Tris-HCl buffer (pH 7.6)	2.0 〃
0.9% KCl	2.5 〃
NADPH	20 μmol
Each snbcellular fraction (3g of liver)	
Total volume	20.0 ml

After treatment of the individual reaction mixtures with Amberlyst A-26, each eluate was fractionated into mono-, di- and trihydroxy bile acid fractions according to Ogura *et al.*[14]

It was demonstrated that the substrate was most effectively converted to the dihydroxy bile acid fraction by the supernant fraction fortified with NADPH and that it was converted almost exclusively to chenodeoxycholic acid, while neither its 7β-epimer (ursodeoxycholic acid) nor any conjugated bile acid was detected by TLC.

I. c. *Metabolism of the Δ^5-Acid with Carp Liver Preparation*

As will be described in the next chapter (II. d), radioactive cholic acid and allocholic acid were isolated from bile of carp which received intraperitoneally the Δ^5-acid-^{14}C.[15] To get further information on the metabolism of the Δ^5-acid in the fish, Yamaga[16] carried out incubation experiments of this acid and its 3-oxo derivative using carp liver preparation.

I. c. i. *Metabolism of the Δ^5-acid*—The Δ^5-acid-^{14}C was incubated with the microsomal fraction of carp liver homogenate in a similar manner to the above experiment (I. b. i). Analysis of the metabolite(s) showed that the substrate was transformed to 7α-hydroxy-3-oxochol-4-enoic acid, suggesting that an isomerase ($\Delta^5 \rightarrow \Delta^4$) activity was present together with a 3β-hydroxysteroid dehydrogenase activity in the preparation, as was not the case with the rat liver preparation. It mignt be noted that such a transformation as mentioned above took place more effectively with NAD than with NADP, while the rat liver preparation required NADP as a preferential cofactor.

Although the Δ^5-acid was converted to cholic acid and allocholic acid in relatively good yields in experiments *in vivo* with carp[15], 12α-hydroxylation of the Δ^5-acid could not be demonstrated with the carp liver preparation.

I. c. ii. *Metabolism of 7α-hydroxy-3-oxochol-4-enoic acid*—7α-Hydroxy-3-oxochol-4-enoic-^{14}C acid was incubated with the supernatant fraction of carp liver homogenate fortified with NADPH-generating system. Thin-layer scannography showed that two radioactive peaks, one (peak I) of which was well coincident with the spot of allochenodeoxycholic acid, but not with that of

chenodeoxycholic acid, the other being corresponding to the starting material (Fig. 4). The resulting product was further purified and identified as allo-chenodeoxycholic acid by isotope dilution technique.

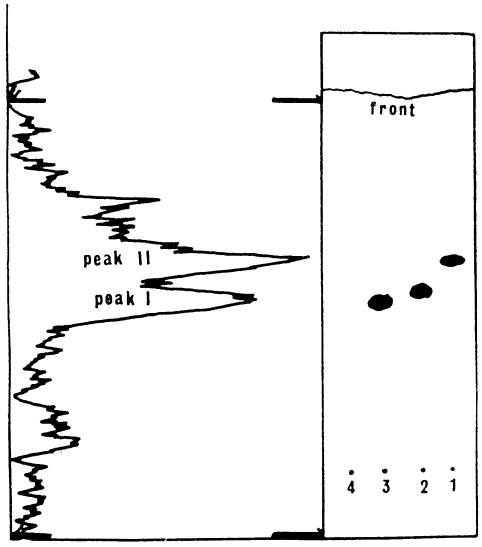

Fig. 4. Thin-layer radioscannography of the products formed by incubation of 7α-hydroxy-3-oxochol-4-enoic-^{14}C acid with the supernatant fraction in the presence of NADPH-generating system. 1, substrate; 2, chenodeoxycholic acid; 3, allochenodeoxycholic acid; 4, incubation products-^{14}C.

II. *Experiments in vivo*

Lindstedt (1957)[17] demonstrated that 7α-hydroxycholesterol is effectively converted to cholic acid and chenodeoxycholic acid in rats with a bile fistula. These bile acids are commonly found in the ratio 1 : 0.25 in rat bile. However, it was later pointed out that the radioactivity ratio of these acids excreted in bile was markedly different from their common biliary ratio, when the rat received 7α-hydroxycholesterol-^{14}C (Danielsson 1961)[18]. Yamasaki and Ogura (1967/70)[19,20] confirmed this indication, as shown in Figs. 5. A and B. Furthermore, they demonstrated that only 40% of the cholic acid fraction was identified as cholic acid itself (Table 5), most of the residual part being proved to be α-muricholic acid, a further oxidized metabolite of chenodeoxycholic acid.[21]

Body and Mawer (1961)[22] isolated 3β-acyl esters of 7α-hydroxycholesterol from the liver and serum of rats. Sakamoto[23] demonstrated by *in vitro* experiments that this dihydroxy sterol was enzymatically acylable like choles-

terol in the presence of the microsomal preparation of the rat liver and that the esters of this sterol were saponifiable with the cholesterol esterase of the liver.

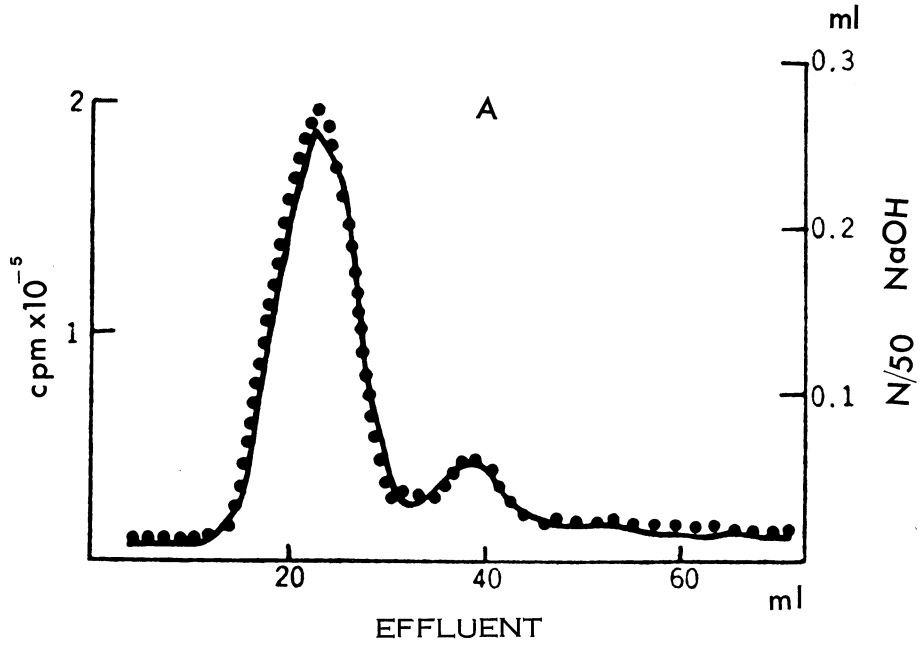

Fig. 5. A. Reversed phase column chromatography of the hydrolyzed bile sample, obtained when cholesterol-^{14}C was administered to a bile-fistula rat. Broken line, radioactivity; solid llne, titration values.

TABLE 5. Cholic Acid-^{14}C Obtained After Administration of Cholesterol-^{14}C and 7α-Hydroxycholesterol-^{14}C to Bile-Fistula Rats.

	Cholesterol-^{14}C	7α-Hydroxycholesterol-^{14}C
Bile sample collected during	24-46 hr	0-12 hr
Cholic acid fraction		
Radioactivity	29,500 cpm	81,300 cpm
Weight	11.3 mg	5.0 mg
Carrier added	100.0 mg	63.6 mg
Specific radioactivity		
Cholic acid isolated	241 cpm/mg	490 cpm/mg
Calculated	265 cpm/mg	1183 cpm/mg
Content of cholic acid-^{14}C	91%	41%

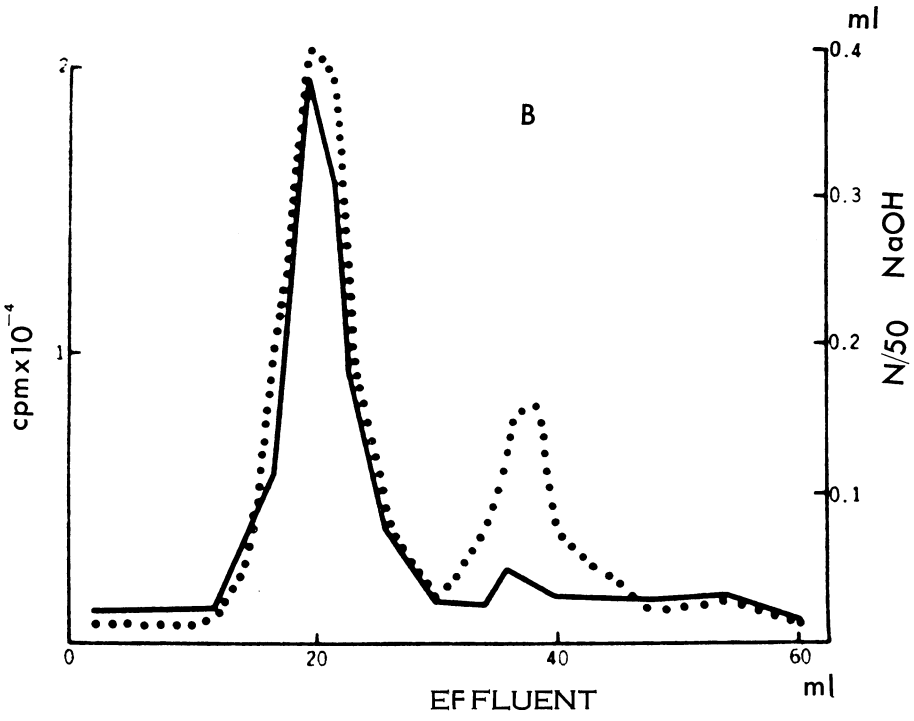

Fig. 5. B. Reversed phase column chromatography of the hydrolyzed bile sample, obtained when 7α-hydroxycholesterol-^{14}C was given to a bile-fistula rat. Other remarks: see Fig. 5. A.

II. a. *Metabolism of 3β-Stearyl-7α-hydroxycholesterol in the Rat*

In order to elucidate the biochemical significance of acyl esters of 7α-hydroxycholesterol, Ogura and Yamasaki[20] administered 3β-stearyl-7α-hydroxy-cholesterol-^{14}C (^{14}C-stearate) to rats with a bile fistula and the bile samples collected at several intervals were analyzed. Individual hydrolyzates of the bile samples were fractionated into the cholic acid and the chenodeoxycholic acid zones by means of TLC to obtain radioactivity ratios of these zones.

When the bile was collected for 6 hr after the stearate-^{14}C was given, radioactivity ratio (1 : 0.86) of these zones was nearly equal to the corresponding ratio (1 : 0.94) obtained when the free dihydroxy sterol-^{14}C was given. However, the bile was collected during the initial 1 hr after the stearate-^{14}C was given, radioactivity ratio (1 : 0.44) of the said zones was close to the common biliary ratio (1 : 0.25)[24]. Such an initial ratio changed gradually with time of collection, reaching the level observed when the free dihydroxy sterol-^{14}C was given.

These findings might be interpreted as follows: The stearate-^{14}C given intraperitoneally reaches the liver cells as such, where it is gradually hydrolyzed like cholesterol esters[25] to yield the free dihydroxy sterol, which, in turn, is metabolized to the bile acids. Since higher fatty acyl esters of 7α-hydroxycholesterol behave chromatographically like free cholesterol (Boyd and Mawer)[22], these esters exogenously administered are expected to be distributed in the subcellular compartments of the liver cells depending on the polarity of individual esters. Hence the stearate-^{14}C is taken up like free cholesterol-^{14}C preferentially by the microsomes (see Table 1), where it is at first hydrolyzed and metabolized *via* the known sequence of the bile acid biogenesis, giving the common biliary ratio of the said zones. But at the later stage of bile collection the stearate-^{14}C is completely hydrolyzed and radioactivity ratio of the two bile acid fractions becomes similar to that observed when the free dihydroxy sterol-^{14}C was given.

The attractive hypothesis of Boyd[26], that cholesterol is catabolized in the form of its linoleate, was proved untenable by the *in vitro* experiments[27]. Free cholesterol-^{14}C was hydroxylated by the incubation with 13,000 *g*— supernatant of rat liver homogenate in the presence of NADPH to give 7α-hydroxycholesterol-^{14}C, whereas such cholesterol-^{14}C esters, as linoleate and the like proved to be hardly hydroxylated by the same incubation procedure as above. Hence it should be rather inferred that the esters of cholesterol and 7α-hydroxycholesterol, contrary to Boyd's assumption, are the reserved, but not activated, forms to be catabolized.

II. b. *Isolation of the Δ^5-Acid and Its By-product (Δ^4-Acid), After Administration of Some Bile-Acid Precursors*

By chance it was observed that the dihydroxy bile acid fraction obtained from the hydrolyzed fistula bile of rats gave a positive Lifschütz reaction for allyl alcohol group on a thin-layer plate.

II. b. i. *Fistula bile of the rat*[28]—Such a ^{14}C-labeled precursor of the bile acid as cholesterol, 7α-hydroxycholesterol or DL-mevalonic acid, was given to bile-fistula rats and the bile sample collected for 6 hr was hydrolyzed. After removal of neutral lipids, the acidic material was fractionated into mono-, di- and trihydroxy bile acid fractions by means of column chromatography[14].

Among the eluates of the dihydroxy bile acid fraction, several portions showed spots on a chromatoplate that gave a positive Lifschütz reaction. These portions of eluates were combined and purified by preparative TLC followed by reversed phase column chromatography to concentrate the Lifschütz-positive fraction, radioscannography of which showed a peak corresponding to the Δ^5-acid (Fig. 6). The fraction so purified was identified as the Δ^5-acid by isotope dilution technique.

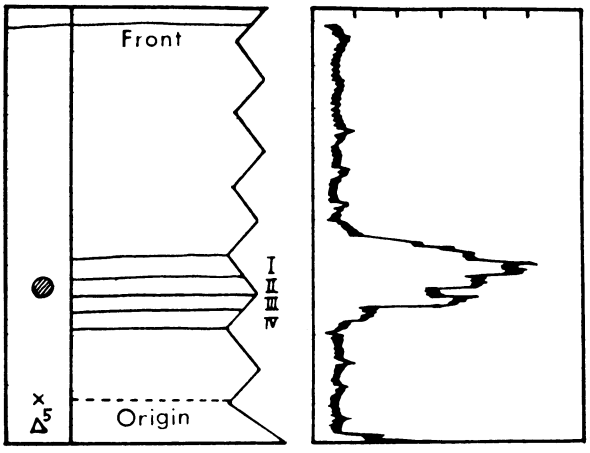

Fig. 6. Thin-layer radioscannography of the Lifschütz-positive fraction obtained from the hydrolyzed bile. I-IV, iodine-stained zones; Δ^5, the Δ^5-acid.

These data showed that the Δ^5-acid was derived not only from cholesterol and 7α-hydroxycholesterol exogenously given but also from endogenous cholesterol formed from mevalonic acid.

II. b. ii. *Fistula bile of the hen*[29]—A hen (white Leghorn) furnished with a bile fistula was injected with 7α-hydroxycholesterol-^{14}C and the bile collected for 7 days was hydrolyzed as usual. The acidic fraction (550 mg) obtained was fractionated portion-wise by preparative TLC on silica gel to give three zones, corresponding to mono-, di- and trihydroxy bile acids, of which the last zone (cholic acid) was unexpectedly much larger than the second zone (chenodeoxycholic acid) unlike the common bile acid pattern of hen bile.

The material eluted from the dihydroxy bile acid zones of several chromatoplates was methylated and rechromatographed to separate the zone corresponding to methyl ester of the Δ^5-acid from the zone of methyl chenodeoxycholate. The eluates corresponding to the Δ^5-acid (methyl ester) were further purified by TLC using two different solvent systems composed of isooctane, ethyl acetate and acetic acid. After the plates were marked with iodine vapor, they were subjected to radioscannography. As shown in Fig. 7. A, the iodine-marked area coincided well with the peak of the radioscannogram. The eluate of this area was rechromatographed on a $AgNO_3$-impregnated plate and marked with iodine vapor followed by radioscannography. As shown in Fig. 7. B, the above-mentioned area sharply divided into two radioactive parts, the upper one (a) corresponding to the Δ^5-acid (methyl ester) and the lower one (b) to methyl 3β, 7α-dihydroxychol-4-enoate (3β-Δ^4-acid). It was later

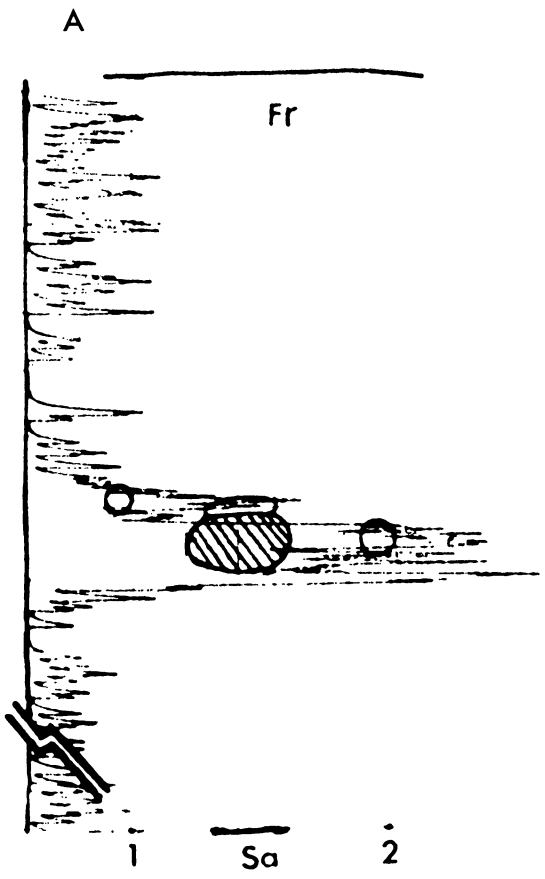

Fig. 7. A. Thin-layer radioscannography of the purified fraction (Sa, methylated, iodine-stained area) corresponding to the Δ^5-acid (methyl ester). 1. methyl chenodeoxycholate; 2, the Δ^5-acid (methyl ester).

(III. b. ii) proved that the material of the lower part was methyl 3α, 7α-dihydroxychol-4-enoate (Δ^4-acid), but not the 3β-Δ^4-acid.

At that time it was reported that the identification of these acids was further confirmed by GLC, compared with the respective authentic samples. Later experiments (IV. a and IV. b) indicated, however, that the respective GLC peaks (t_R : 5-6 min) concerned corresponded to those of the decomposed compounds derived from the Δ^5-acid and the Δ^4-acid. Although these data were consequently ambiguous, the deduction was fortunately correct (III. b).

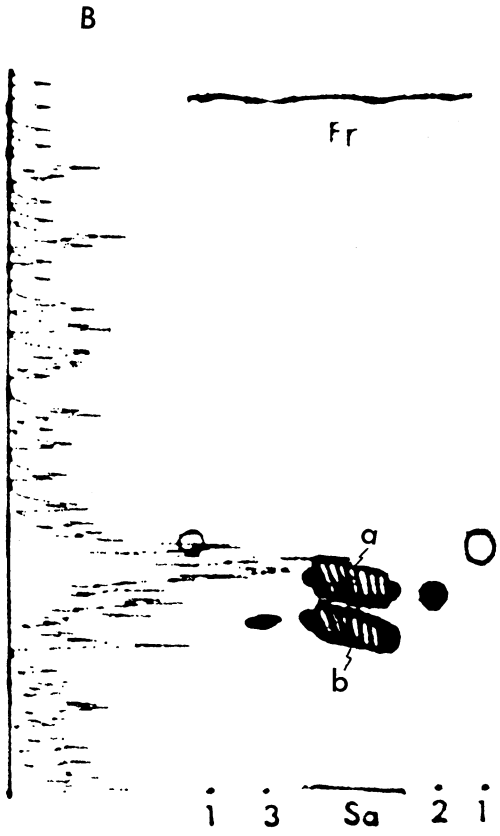

Fig. 7. B. Thin-layer radioscannography of the same fraction (Sa), using a $AgNO_3$-impregnated plate. 1, methyl chenodeoxycholate: 2, the Δ^5-acid (methyl ester); 3, the 3β-Δ^4-acid (methyl ester).

II. c. *Metabolism of the Δ^5-Acid*

Based on the findings that 7α-hydroxycholesterol is converted to the primary bile acid[17] and that it was dehydrogenated to a 3-oxo compound, probably 7α-hydroxycholest-4-en-3-one, by a specific enzyme (system) in the rat liver, the author and his associates (1958/59)[30] advanced a hypothesis that the above mentioned 3-oxo compound is an obligatory metabolite in the bile acid biogenesis. Experimental data[18,31] have later been reported in favor of the hypothesis.

II. c. i. *Metabolism of the Δ^5-acid*[32]—As an analogue of 7α-hydroxycholesterol, the ^{14}C-labeled Δ^5-acid was administered intraperitoneally to rats furnished with a bile fistula and almost all of the radioactivity given was recovered in bile samples collected within 20 hr.

The sample was hydrolyzed as usual and the acidic fraction was subjected to reversed phase column chromatography. Two radioactive peaks I and II were observed corresponding to the cholic acid and chenodeoxycholic acid fractions, as shown in Fig. 8.

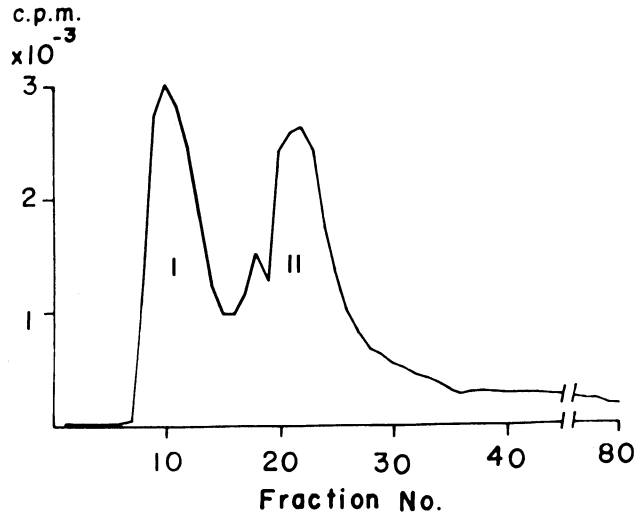

Fig. 8. Reversed phase column chromatography of the bile acids extracted from the hydrolyzed bile of a fistula rat which received the Δ^5-acid-^{14}C.

The fraction corresponding to peak II was identified as chenodeoxycholic acid by isotope dilution technique and about 84 % of radioactivity of this fraction was contained in the chenodeoxycholic acid molecule.

The radioactive metabolite corresponding to peak I was not identified as cholic acid and suggested to be a muricholic acid. Later Ikawa and Yamasaki[33] demonstrated that the metabolite concerned was α-muricholic acid contaminated with a small amount of β-muricholic acid.

II. c. ii. *Metabolism of 7a-hydroxy-3-oxochol-4-encic acid*[13]—The title bile acid is an analogue of the 3-oxo compound derived from 7α-hydroxycholesterol[30]. After 24 hr canalization of the bile duct of rats furnished with a bile fistula, an emulsified solution of the title acid-^{14}C was injected intraperitoneally. The bile sample collected for initial 6 hr was treated with Amberlyst A-26 and about 40 % of the radioactivity given was recorvered. An aliquot of the eluate showed a single prominent radioactive peak corresponding to taurochenodeoxycholate on a chromatoplate. But the acidic fraction obtained by hydrolysis of the residual eluate showed two radioactive peaks

on a chromatoplate, the major one (peak I) corresponding to chenodeoxycholic acid and the other (peak II) to cholic acid. The quantitative ratio of these was about 4 : 1.

By isotope dilution technique the metabolite corresponding to peak I was mainly identified as chenodeoxycholic acid, its small part being identified as ursodeoxycholic acid.

The metabolites corresponding to peak II were fractionated by preparative TLC after methylation. Radioactivity was found in the zones of methyl α- and β-muricholic acids, especially in the former. Identification of the respective acids was performed successfully by isotope dilution technique.

II. d. *Metabolism of the Δ^5-Acid in the Carp*

It has been demonstrated[34] that chenodeoxycholic acid is converted to cholic acid in eel, suggesting that 12α-hydroxylation of the steroid molecule takes place in spite of the current view of cholic acid biogenesis, even after the side-chain of cholesterol or its C_{27}-derivatives is cleaved.

Yamaga[15] investigated whether such a 12α-hydroxylation generally occurs in fish, when the Δ^5-acid-^{14}C is given to carp. He also aimed to obtain some line of evidence for the conversion of the Δ^5-acid to any 5α-bile acid(s), because 5α-cyprinol sulfate is the major bile salt of this fish.[1]

The Δ^5-acid-^{14}C was injected as an emulsified solution intraperitoneally in two carps. These fish were kept in a water vessel for 1 week without feeding. The collected bladder bile contained about 60 % of the radioactivity given. It was hydrolyzed as usual and, after removal of neutral lipids, the hydrolyzate was acidified and extracted with ether to obtain the acidic fraction. Radioscannography as well as reversed phase column chromatography showed two radioactive peaks I and II, corresponding well to the cholic acid and chenodeoxycholic acid fractions, the quantitative ratio of these being about 1.7 : 1.0. It must be noted that peak II of the titration curve (Fig. 9) was very faint, which was well in accordance with the finding that any dihydroxy bile acid has not yet been isolated from carp bile.

The material corresponding to peak I was fractionated into two main zones by TLC. The respective zones extracted from the plate were identified by isotope dilution technique as cholic acid and allocholic acid, respectively. TLC followed by scannography of the dihydroxy bile acid fraction showed also two zones corresponding to chenodeoxycholic acid and allochenodeoxycholic acid. The metabolite in the lower zone of the plate was identified as chenodeoxycholic acid by isotope dilution technique. Although the upper zone was well coincident with the spot of allochenodeoxycholic acid (Fig. 10), no further evidence for identification was then lacking. However, 7α-hydroxy-3-oxochol-

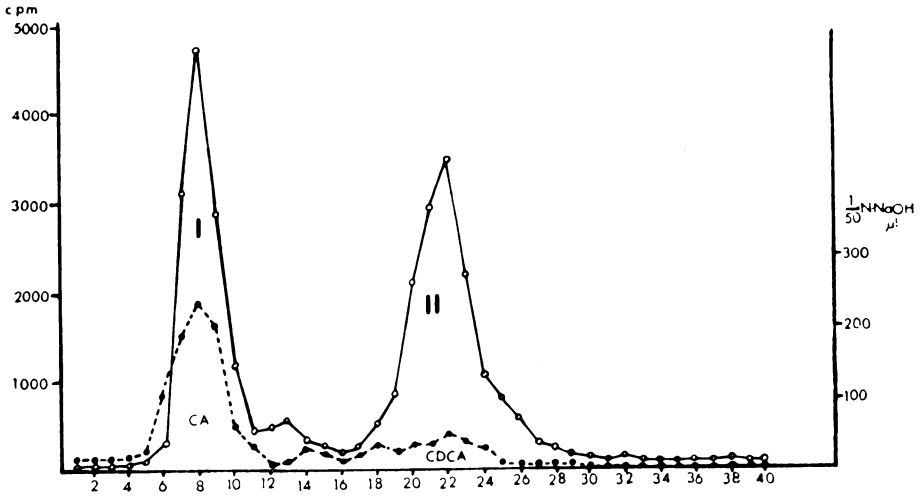

Fig. 9. Reversed phase column chromatography of the hydrolyzed bile obtained from a carp received the Δ^5-acid-^{14}C. Broken line, titration values; solid line, radioactivity. CA, cholic acid; CDCA, chenodeoxycholic acid.

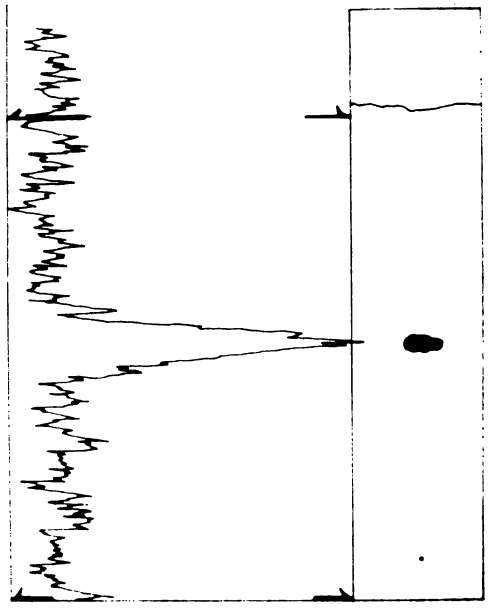

Fig. 10. Thin-layer radioscannography of the extract of the upper zone obtained by TLC of the fraction corresponding to peak II in Fig. 9. Reference sample: allochenodeoxycholic acid.

4-enoic acid was converted to allochenodeoxycholic acid by incubation with the supernatant fraction of carp liver homogenate[16], as described above (I. c. ii).

II. e. *Metabolism of 7β-Hydroxycholesterol*

Yamasaki *et al.* (1958/59)[30] have once advanced another hypothesis that the 7α-hydroxyl group of 7α-hydroxycholesterol, a key intermediate of the bile acid biogenesis, is responsible for the stereospecific transformation of the nuclear part of cholesterol (see II. c), because Harold *et al.*[34] showed that 3-oxocholest-4-ene was readily converted to 5α-cholestan-3β-ol, but not to any natural bile acid *in vivo* as well as *in vitro*. Several data favorable for the hypothesis have hitherto been reported by the author's group[32,36,37]. Björkem *et al.* (1968)[38] demonstrated that the 7β-epimer of the hydroxycholesterol concerned was not converted to any naturally occurring bile acid in rats with a bile fistula. In order to get further information of the stereospecific transformation of cholesterol to the C_{24}-bile acids and to know whether 7β-hydroxycholesterol is a precursor of ursodeoxycholic acid, 7β-epimer of chenodeoxycholic acid, Norii *et al.* (1970)[39], independently of the Swedish workers[38], administered the ^{14}C-labeled 7β-hydroxycholesterol to rats fitted with a bile fistula and analyzed the bile sample collected. Most part (94 %) of the radioactivity given was excreted in bile within 5 days.

The bile sample was hydrolyzed as usual and after removal of neutral lipids, the hydrolyzate was acidified and extracted with ether to obtain the acidic fraction. Reversed phase column chromatography of the acidic product showed two major peaks of radioactivity. The one (peak I) was found between the cholic acid and chenodeoxycholic acid zones and the other (peak II) in a zone slightly less polar than that of chenodeoxycholic acid, as shown in Fig. 11.

Rechromatography of the respective fractions corresponding to peaks I and II showed that neither of these corresponded to the titration peak of ursodeoxycholic acid added as a carrier, but that they were well coincident with the respective titration peaks of 3β, 7β-dihydroxychol-5-enoic acid (3, 7-DiOH Δ^5-acid) and 3β-hydroxy-7-oxochol-5-enoic acid (3-OH-7-oxo Δ^5-acid) added as carriers. Then they were subjected to isotope dilution experiments using the respectively possible epimers as carriers. The individual yields (%) in the fractions corresponding to peaks I and II were calculated, as follows.

Peak I (3, 7-DiOH Δ^5-acid)		*Peak II (3-OH-7-oxo Δ^5-acid)*
3β, 7β- 34.0%	3α, 7β- 45.3%	3β-OH, 7-oxo- 6.7%
3β, 7α- 8.7	3α, 7α- 8.1	3α-OH, 7-oxo- 72.2
(total 96.1%)		(total 78.9%)

The effuents collected from between peaks I and II (Fig. 11) were extracted with ether on acidification. Isotope dilution experiments of the extract

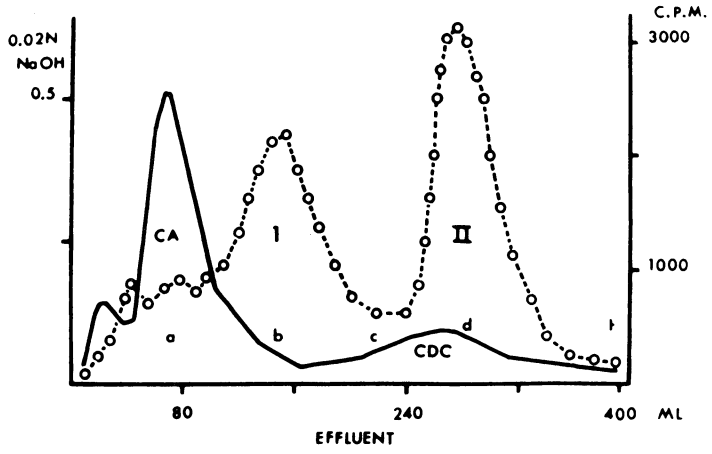

Fig. 11. Reversed phase column chromatography of the acidic fraction obtained from the hydrolyzed bile of a fistula rat which received 7β-hydroxycholesterol-^{14}C. Broken line, radioactivity; solid line, titration values. CA, cholic acid; CDC, chenodeoxycholic acid.

showed that only a small percentage of its radioactivity was found in both molecules of ursodeoxycholic acid and chenodeoxycholic acid.

II. f. *Metabolism of the 7β-Epimer of the Δ^5-Acid*

Recently Ikawa[40] made the following interesting observations, when the 7β-epimer-^{14}C of the Δ^5-acid (the side-chain cleaved product of 7β-hydroxycholesterol) was administered to rats fitted with a bile fistula. (i) It was excreted in bile much more rapidly than the metabolites of 7β-hydroxycholesterol-^{14}C; (ii) this acid was largely converted to 3β, 7β-dihydroxychol-5-enoyl taurine and its sulfate in nearly equal portions, the nuclear part of its molecule being unchanged.

The major metabolite in the non-sulfated fraction was identified as 3β, 7β-hihydroxychol-5-enoyl taurine together with a ltttle tauroursodeoxycholate and unidentified metabolites, while any 3-hydroxy-7-oxochol-5-enoic acid (II. e.) was not identified as such.

Although Norii *et al.*[39] and Ikawa[40] made the observations somewhat different from each other, they equally suggested strongly that the 7α-hydroxyl group attached to the steroid molecules plays an important role in the biogenesis of the natural bile acids in the rat.

III. *Isolation of the Δ^5-Acid and Δ^4-Acid from Human and Hen Bile*

From the foregoing data obtained from the experiments *in vitro* and *in vivo*, it could be concluded that the alternative pathway proposed by the

author's group takes place in the rat and hen. Actually the Δ^5-acid and its isomer, Δ^4-acid, were isolated from human and hen biles and identified as such by some modern techniques, such as gas chromatography-mass spectrometry (GC-MS), NMR spectrometry and the like.

III. a. *From Fistula Bile of Humans*

Human fistula bile (4 liters) was collected from some patients suffering from cholelithiasis. After the sample was hydrolyzed as usual, the acidification and extraction (with ethyl acetate) were conducted so carefully as to minimize the possibility of attack by H^+, because both the Δ^5-acid and its Δ^4-isomer have an acid-labile allyl alcohol group in each molecule. The extract was condensed to give a crystalline material (mainly cholic acid, 11 g). The filtrate was further condensed to a small volume and methylated.

Preparation of the Lifschütz-positive compounds (LPC)—The methylated material was subjected to column chromatography and each eluate was checked by the Lifschütz reagent to obtain the Lifschütz-positive eluates (LPC). An aliquot of LPC showed one spot on an ordinary plate, but when it was developed on a $AgNO_3$-impregnated plate, two spots were observed, corresponding to the respective methyl esters of the 3β-Δ^4-acid and Δ^5-acid (Fig. 12). As will be described in the next chapter, they were methoxylated in methanol containing a trace of HCl in order to insure against heat decomposition on GC-MS and then subjected to column chromatography. The Lifschütz-positive methoxylated eluates (m-LPC) were combined and proved to be almost completely free from methyl chenodeoxycholate, as shown in Fig. 12.

GC-MS of m-LPC—After acetylation, the material (m-LPC) thus obtained was subjected to GC-MS, compared with the mass spectra taken, after acetylation, from the 3β-methoxylated* and 7α-methoxylated compounds derived from the respective methyl esters of 3β, 7α-dihydroxychol-4-enoic acid (3β-Δ^4-acid) and the Δ^5-acid. The fraction of t_R 15 min and that of t_R 20 min obtained from m-LPC, though GC peak of the latter was not so high, showed the fragmentation patterns (m-LPC_1 and m-LPC_2), as shown in Fig. 13. They were not only similar to the respective patterns taken from the authentic methoxylated samples, but also almost similar to each other. However, it must be noted that the fragmentation ion m/e 138 (Chart B, c) was observed on both spectra taken from the 3β-methoxylated compound of the 3β-Δ^4-acid* and the fraction of t_R 15 min, but not on those taken from the 7α-methoxylated compound of the Δ^5-acid and the fraction of t_R 20 min and that the fragmentation ion 400 m/e, which was common and characteristic on both spectra (m-LPC_1 and m-LPC_2), was of different structure (Chart B, a and b).

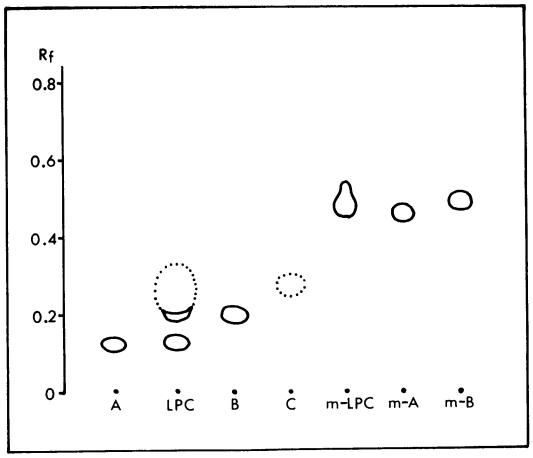

Fig. 12. TLC of the partially purified fraction (LPC) obtained from the hydrolyzate of human bile and its methoxylated compound (m-LPC), using a $AgNO_3$-impregnated plate. A, B, m-A and m-B: 3β-Δ^4-acid (methyl ester), Δ^5-acid (methyl ester) and their methoxylated compounds; C: methyl chenodeoxycholate.

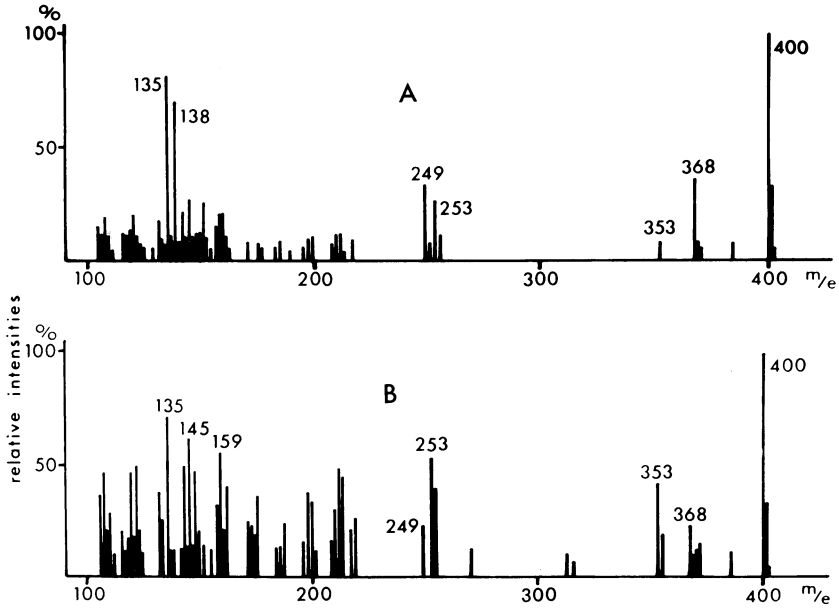

Fig. 13. GC-MS spectra (m-LPC_1: A and m-LPC_2: B) taken from the methoxylated compounds of LPC.

From these data it would be concluded that the Lifschütz-positive acids (LPC) found in human bile are the Δ^5-acid and Δ^4-acid.*

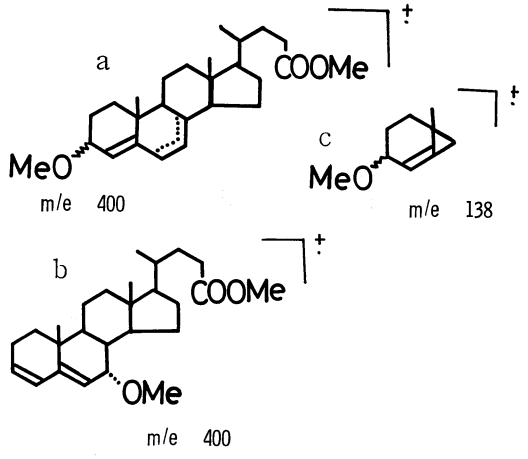

Chart B Fragmentation ions derived from the methoxylated compounds (methyl ester) of the Δ^4-acid and the Δ^5-acid.

III. b. *From Bladder Bile of Hens*

From the bile of domestic fowls, so far, five bile acids have been isolated, chenodeoxycholic, cholic, allocholic, 3α-hydroxy-7-oxocholanoic and 3-oxochol-4, 6-dienoic acids, of which chenodeoxycholic acid is the major component (see Ref. 42).

Some years ago Yamasaki and Yamasaki[42] isolated a Lifschütz-positive bile acid (LPC-I) from bladder bile of hens, to which the structure 3β, 7α-dihydroxychol-4-enoic acid (3β-Δ^4-acid) was assigned, and assumed that it was a by-product of the alternative pathway proposed by the author's group[9].

Another Lifschütz-positive compound (LPC-II) was also detected in the same specimen, but no further investigation was then performed. On the basis of its far less polar localization on a chromatoplate, it might be suggested that LPC-II was an artifact, *i.e.*, an alkoxy compound derived from the Δ^5-acid during the experimental procedures, because it has an acid-labile allyl alcohol group in the molecule like 7α-hydroxycholesterol.

III. b. i. *Isolation of the Δ^5-acid*—Lately Ikeda and Yamasaki[43] attempted to isolate the Δ^5-acid itself from bladder bile of hens. Bile sample (2.4 liters)

* Ikeda and Yamasaki[43] demonstrated later that the methoxylated compounds of methyl 3α, 7α- and 3β, 7α-dihyroxychol-4-enoates (Δ^4-acid and 3β-Δ^4-acid) retained mostly the original configurations and that the mass patterns of fragmentation ions taken from both epimers are the same (see III. b. ii).

of hens was hydrolyzed as usual. Since the Δ^5-acid, if present, is acid-labile, the hydrolyzate was carefully treated so as to minimize the possibility of attack by H^+. The hydrolyzate was neutralized (pH 6–7) with 2N hydrochloric acid under cooling; immediately after, 10 % ammonium hydroxide solution and 10 % barium chloride solution were alternately added (pH 9) to precipitate chenodeoxycholic acid (Ba salt) as completely as possible. The precipitate was treated to remove most part of Ba salt of chenodeoxycholic acid together with Ba salts of fatty acids by repeated recrystallizations. From the combined filtrates a crystalline material was obtained which gave a strong Lifschütz-positive reaction. This material was extracted with ethyl acetate on careful acidification after conversion to sodium salt. The extract was separated into mono- and dihydroxy bile acid fractions according to Ogura *et al.*[14] The resulting dihydroxy acid fraction (Lifschütz-positive compounds, LPC) was worked up in two ways: one part was used for isotope dilution technique (Exp. 1) and the other for GLC (Exp. 2).

Experiment 1—After methylation the one part was chromatographed on a silicic acid column and then on a column of $AgNO_3$-impregnated silica gel using chloroform to separate two fractions, compound A and compound B, corresponding to methyl esters of the Δ^5-acid and 3β-Δ^4-acid, respectively (compound B will be described in the next section, III. b. ii). After acetylation with ^3H-acetic acid-pyridine, compound A was cocrystallized with acetyl methyl ester of the Δ^5-acid to constant radioactivity and proved to be mainly composed of methyl ester of the Δ^5-acid.

Experiment 2—In order to confirm the above finding, the other part of LPC was methylated and then methoxylated as will be described in the next chapter (IV. a. ii). The product was chromatographed to collect the Lifschütz-positive fraction. This fraction was acetylated and crystallized from aqueous acetone to give a crystalline material, which was identified as acetyl methyl ester of 7-oxo-lithocholic acid[44] by means of GLC. Most of the remaining ester of the 7-oxo acid was removed by crystallization and filtration. GLC of the material obtained from the filtrate gave several peaks, as shown in Fig. 14, A. Among these, peak d, though rather low in height, showed the same relative retention time (RRT) as the methoxylated acetyl methyl ester of the Δ^5-acid (Table 6). This finding was further confirmed by GLC after adding an authentic sample of methoxylated acetyl methyl ester of the Δ^5-acid (Fig. 14, B).

As will be described below (IV. b), methyl ester of the Δ^4-acid is also readily methoxylated by methanol-H^+ treatment. Table 6 shows that RRT of peak c (Fig. 14, A) is well coincident with that of the methoxylated compound derived from the Δ^4-acid.

TABLE 6. Relative Retention Times (RRT) with Respect to Methyl Lithocholate (Peak a, t_R: 6.2 min).

	RRT
Acetyl methyl ester of 3α-methoxy-7α-hydroxychol-4-enoic acid[a]	1.35
Peak c	1.35
Acetyl methyl ester of 3β-hydroxy-7α-methoxychol-5-enoic acid[b]	1.73
Peak d	1.76

a This was derived from compound B (III. b.ii) by refluxing in methanol-acetic acid.
b This was prepared from methyl 3β,7α-dihydroxychol-5-enoate by incubation in methanol-HCl followed by acetylation.

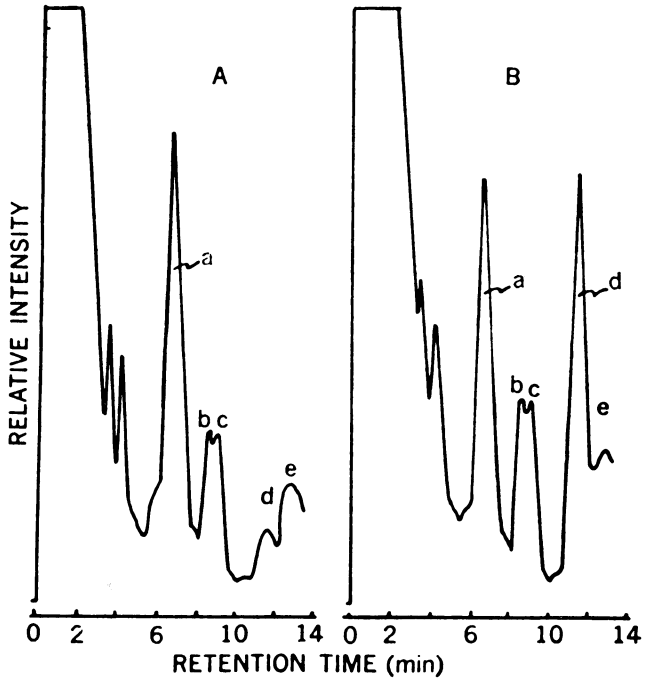

Fig. 14. GLC of the filtrate of 7-oxo-lithocholic acid (acetyl methyl ester). Methyl lithocholate was used as an internal standard (peak a, t_R: 6.2min). Chromatogram A: the filtrate; chromatogram B: the filtrate mixed with the methoxylated Δ^5-acid (acetyl methyl ester). Peaks c and d correspond to acetyl methyl esters of the methoxylated Δ^4-acid and Δ^5-acid, respectively (see Table 6). Peaks b and e remain undertermined. For convenience the peak (RRT: 2.06) corresponding to 7-oxo-lithocholic acid (acetyl methyl ester) is omitted.

III. b. ii. *Isolation of the Δ^4-acid*—As mentioned above, Yamasaki and Yamasaki[42] for the first time isolated an allylic (Lifschütz-positive) bile acid

from bladder bile of hens and assigned the structure 3β, 7α-dihydroxychol-4-enoic acid (3β-Δ^4-acid).

Compound B mentioned above (III. b. i) was obtained in an amount sufficient for further study. It showed almost the same mobility as methyl ester of the 3β-Δ^4-acid on an ordinary chromatoplate as well as on a $AgNO_3$-impregnated plate. However, it proved not only non-precipitable with a digitonin solution, but also its melting point (mp 142-144°C) was markedly depressed on admixture with methyl ester of the 3β-Δ^4-acid (mp 143-145°C), but not with its 3α-epimer (Δ^4-acid, mp 143-144°C). These findings strongly indicate that compound B is methyl 3α, 7α-dihydroxychol-4-enoate (Δ^4-acid) but not its 3β-epimer (3β-Δ^4-acid). This was confirmed by further experiments, such as mass and NMR spectrometries and molecular rotation difference, compared with methyl ester of the 3β-Δ^4-acid.[43]

IV. *Methoxylation of the Δ^5-Acid and Its Isomers*

IV. a, *Methoxylation of the Δ^5-Acid*

IV. a. i. *Nature of peak IV (I. a. i.) and peak III (I. a. ii)*—When 7α-hydroxycholesterol-^{14}C was incubated with the mitochondrial fraction of the rabbit liver (I. a. ii), the acidic fraction obtained showed three radioactive peaks (Fig. 2) on reversed phase column chromatography. Two (peaks I and II) of these were proved to correspond to the Δ^5-acid and chenodeoxycholic acid, respectively. The least polar peak (peak III) was then assumed to correspond to such an artifact as a 7-methoxy-compound derived from the Δ^5-acid, as is the case with 7α-hydroxycholesterol.[44] This assumption was based on the observation that the peak concerned was well coincident with the third titration peak, which corresponded to an artifact presumably derived from the Δ^5-acid added as a carrier.

The experimental procedures then adopted (I. a. ii) provided unconsciously the conditions for methoxylation of the Δ^5-acid: first, the extraction of the acidic metabolites of 7α-hydroxycholesterol-^{14}C with ether was performed on acidification (HCl) of the hydrolyzate obtained from the incubation mixture; second, the extract, which contained inevitably a trace of HCl, was dissolved in methanol to be subjected to reversed phase column chromagraphy, using solvent system FI, one component of which is dilute methanol.

When 7α-hydroxycholesterol-^{14}C was incubated with the mitochondrial fraction of the rat liver (I. a. i), an unknown radioactive peak IV, was observed in a region less polar than that of chenodeoxycholic acid like peak III (I. a ii; see Figs. 1 and 2).

The assumption, that both peak III and peak IV corresponded to a 7-

methoxy-compound of the Δ^5-acid-^{14}C derived from 7α-hydroxycholesterol-^{14}C incubated, was substantiated by the results described in the next section.

IV. a. ii. *Methoxylation of the Δ^5-acid, compared with its 7β-epimer.*— Methyl esters of the Δ^5-acid and its 7β-epimer were incubated in methanol containing a trace of HCl at room temperature and the products were monitered at various incubation times by TLC and GLC (Fig. 15; Fig. 16).

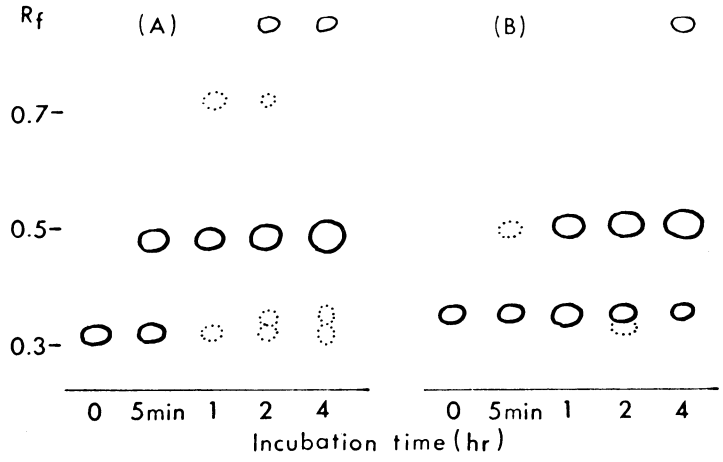

Fig. 15. TLC of the respective compounds obtained from methyl esters of the Δ^5-acid (A) and its 7β-epimer (B), when they were incubated in methanol-HCl for various times.

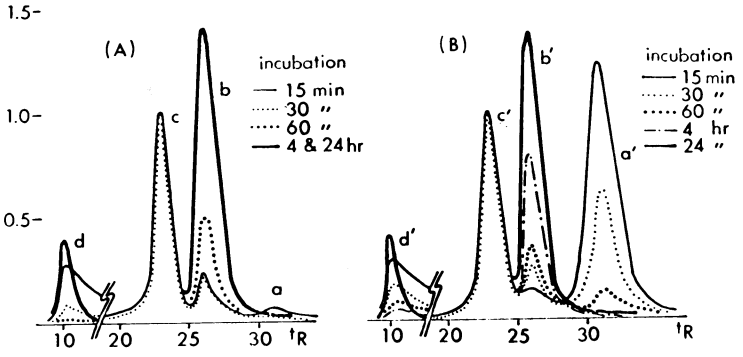

Fig. 16. GLC of the same respective compounds as detected by TLC (Fig. 15). Heights of peak c and c' are taken as the standard (1.0) of the respective chromatograms.

GLC of the individual products showed two distinct peaks, b (b') and c (c'), which corresponded to the respective single spots (R_f 0.5) detected by TLC.

In accordance with the results of TLC, methyl ester of the Δ^5-acid (peak a) disappeared promptly, but its 7β-epimer (peak a′) was still observed on the GLC chart even at 1 hr incubation. This indicates that methoxylation of the former proceeds much faster than that of the latter.

Methyl ester of the Δ^5-acid was incubated for 1 hr and that of its 7β-epimer for 4 hr to isolate the respective products. The major product obtained from methyl ester of the Δ^5-acid was its 7α-methoxy-compound (peak c) together with its 7β-methoxy-compound (peak b), while the 7β-epimer of the Δ^5-acid gave its 7β-methoxy-compound (peak b′) as the major product, accom panied with its 7α-methoxy-compound (peak c′) and a small amount of methyl 3β-hydroxy-5, 7-dienoate (peak d′).

IV. b. *Methoxylation of the Δ^4-Acid, Compared with Its 3β-Epimer*

When methyl esters of the Δ^4-acid and its 3β-epimer (3β-Δ^4-acid) were refluxed in methanol-acetic acid (10 : 1, by vol.), they were converted to 3-methoxy-compounds on an analogy of 7-hydroxycholesterols, the former giving a mixture of compounds I and II by 1 hr refluxing and the latter giving a mixture of compounds III and IV by 4 hr refluxing together with a trace of methyl 7α-hydroxychol-3, 5-dienoate. Physical as well as spectral data showed that compound I and compound IV were of the same structure (3α-methoxy-compound of the Δ^4-acid), while compound II and compound III were of the same structure (3β-methoxy-compound of the Δ^4-acid) (Tables 7 and 8). The GLC data showed that the original configurations of C_3-OH in both mother epimers mostly retained on methoxylation, small parts being epimerized (Fig. 17; Table 7).

TABLE 7. Physical Data of the Methoxy-Compounds Derived from Methyl 3α, 7α-Dihydroxychol-4-enoate and Its 3β-Epimer.

Compound	mp (°C)	$[\alpha]_D$(MeOH)	R_f(TLC)	RRT	Yield(%)
I	67- 68	+66.5°	0.36	1.40	55.8
II	100-101	——	0.43	1.50	10.2
III	98- 99	+11.5°	0.43	1.51	50.5
IV	67- 68	+76.1°	0.35	1.41	17.2

When the Δ^5-acid and the Δ^4-acid were subjected to the conventional methods of GLC or mass spectrometry (MS) in the bile acid field, neither of these procedures afforded any definite information for their identification due to thermal decomposition of the respective allyl alcohol groups. This is why these biliary unsaturated bile acids have not hitherto been found in any bile samples by GLC or MS[48], even though they were proved by isotope dilution

technique to be the metabolites of endogenous cholesterol-^{14}C as well as of cholesterol-^{14}C and 7α-hydroxycholesterol-^{14}C given to the rat[28] and hen[29].

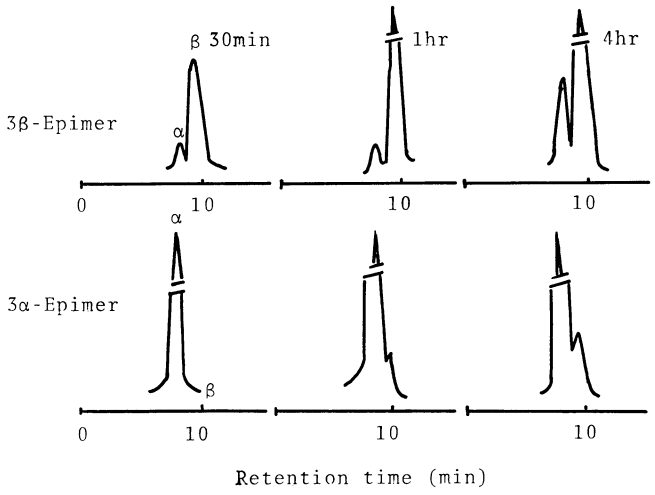

Fig. 17. GLC of the methoxylated compounds (without acetylation) derived from methyl esters of the Δ^4-acid (3α-Epimer) and its 3β-epimer (3β-Epimer) at various refluxing times.

TABLE 8. Coupling Constants (4H-3H) and Molecular Rotation Difference (ΔM_D) of 3α- and 3β-Methoxy-compounds Derived from Methyl 3α, 7α-Dihydroxychol-4-enoate (3α-Epimer) and Its 3β-Epimer, Compared with Those of the Mother Epimers.

Cmpound	Coupling Constant*	M_D	ΔM_D
3α-Methoxy-compound (Compounds I ahd IV)	4H-3βH: δ: 5.61, 5.56; J=5 Hz	+318.9°	+270.7°
3β-Methoxy-compound (Compound III)	4H-3αH: δ: 5.46; J≒ 0 Hz	+ 48.2°	
Mother 3α-Epimer	4H-3βH: δ: 5.51; J= 5 Hz	+188°	+120°
Mother 3β-Epimer	4H-3αH: δ: 5.40; J≒ 0 Hz	+ 68°	

* NMR spectra were observed on a Hitachi R-22 spectrometer (90 MHz).

Taking advantage of the experimental data mentioned above (IV. a. ii ; IV. b), the Δ^5-acid and the Δ^4-acid were isolated from human and hen biles and identified as such by means of GLC, MS or GC-MS, after they were methoxylated in advance, as described above (III. a ; III. b).

It should be noted that mass spectrometry of both methoxy-compounds showed similar patterns of fragmentation ions to each other and the same characteristic, but structurally different, ions m/e 400 (Chart B, a ; b) and that the ion m/e 138 (Chart B, c) was found on the spectrum of the methoxy-compound of the Δ^4-acid, but not on that of the methoxy-compound of the Δ^5-acid (Fig. 13).

B. BIOGENESIS OF CHENODEOXYCHOLIC ACID *VIA* 3β-HYDROXY-CHOL-5-ENOIC ACID

Bergström (1955)[24] preliminarily reported that the major metabolic product of 3β-hydroxychol-5-enoic acid in the rat was similar to, but different from, cholic acid. Later Mitoropolous and Myant (1966/67)[8] reported that, when cholesterol-^{14}C was incubated with rat liver homogenate it gave 3β-hydroxychol-5-enoic acid, which, in turn, was metabolized *via* lithocholic acid to various natural bile acids except for cholic acid, suggesting the title pathway of chenodeoxycholic acid biogenesis.

Independently of the British authors, Nakada *et al.*[49] and later Ikawa and Yamasaki[33] showed that 3β-hydroxychol-5-enoic acid and its esters (acetate and methyl ester) were converted not only to such natural bile acids as reported by the British authors, but also to some unnatural bile acids of 5α-

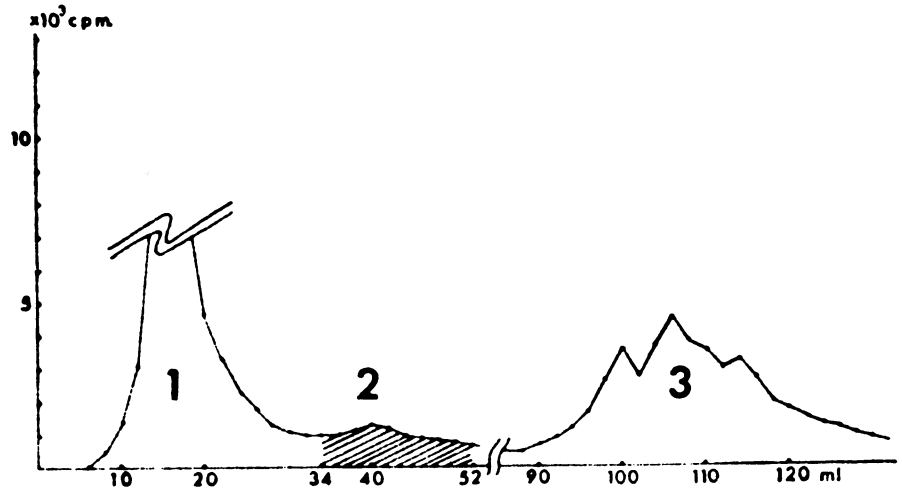

Fig. 18. Reversed phase column chromatography of the acidic fraction obtained from the hydrolyzed bile of a fistula rat which received 3β-hydroxychol-5-enoic-^{14}C acid (acetate). 1, 2 and 3 : tri-, di- and mono-hydroxy acid zones.

series. Moreover, reversed phase column chromatography of the metabolites from 3β-hydroxychol-5-enoic-^{14}C acid showed that the dihydroxy acid peak was so faint (Fig. 18), compared with those of the mono- and trihydroxy acids, that this unsaturated acid was scarcely be assumed as a natural precursor of chenodeoxycholic acid, one of the primary metabolites of cholesterol.

Based on these data including those shown in Table 9, the metabolic pathway of 3β-hydroxychol-5-enoic acid in the rat should be modified as follows: 3β-hydroxychol-5-enoic acid $\longrightarrow$ lithocholic acid $\longrightarrow$ 3α,6β-dihydroxy-5β-cholanoic acid $\longrightarrow$ β-muricholic acid. Furthermore, it must be noted that the main metabolite(s) of lithocholic acid is different from species to species, as shown in Table 9.

TABLE 9. Metabolism of Lithocholic Acid.

Animal Species	Metabolite	Experiment
Rat	3α,6β-Dihydroxy-5β-cholanoic acid (mainly); chenodeoxycholic acid	*in vitro*[1]
Rat	3α,6β-Dihydroxy-5β-cholanoic acid; α- and β-muricholic acids	*in vivo*[2]
Mouse	3α,6β-Dihydroxy-5β-cholanoic acid; an unidentified trihydroxy acid(s)	*in vivo*[3]
Dog	Chenodeoxycholic acid	*in vivo*[3]
Chicken	3α,6β-Dihydroxy-5β-cholanoic acid	*in vivo*[4]
Hog	Hyodeoxycholic acid	*in vitro*[5]
Rabbit	Not hydroxylated	*in vivo*[4]
Human	Hydroxylated (only 2-3%)	*in vivo*[6]

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If 3β-hydroxychol-5-enoic acid were an immediate precursor of lithocholic acid, it should be expected that the epimerization of its 3β-hydroxyl group took place, 3-oxochol-4-enoic acid being a possible intermediary product. However, it has not yet been successful in experiments *in vitro* and *in vivo* with rats, that this 3-oxo-Δ^4-acid was converted to chenodeoxycholic acid, though it was mainly converted to lithocholic acid* together

* It was reported that 3-oxochol-4-enoic-^{14}C acid was converted mainly to lithocholic acid, when it was given to male rats.[37] However, allolithocholic acid was the major metabolite, when the 3-oxo-Δ^4-acid-^{14}C was given to female rats (Cronholm, T., Makino, I. and Sjövall, J.: Europ. J. Biochem. **26,** 251, 1972).

with a product which was similar to, but different from, chenodeoxycholic acid.

From these data it might be concluded that the contribution of 3β-hydroxychol-5-enoic acid as a precursor of chenodeoxycholic acid is negligible, compared with that of 3α, 7α-dihydroxy-5β-cholestane and that of 3β, 7α-dihydroxychol-5-enoic acid. However, it has lately been demonstrated that cholesterol seems to be metabolized through 3β-hydroxychol-5-enoic acid in a measurable amount in some pathological conditions, such as biliary atresia and some other hepatobiliary diseases of human. In such cases, this unsaturated acid and its possible metabolites, lithocholic acid and allolithochlic acid, were partially sulfated and excreted in urine.[51]

DISCUSSION AND CONCLUSION

When 7α-hydroxycholesterol-^{14}C and cholesterol-^{14}C were administered intraperitoneally to bile-fistula rats, their distributions in the subcellular compartments of the liver cells were characteristically different from each other, probably due to their own polarities. 7α-Hydroxycholesterol-^{14}C* was taken up preferentially by the mitochondrial fraction, while most part of cholesterol-^{14}C* was found in the microsomal fraction of the liver homogenate[20] (Table 1). As was expected, the pattern of the bile acids derived from cholesterol-^{14}C given was proved to be the same as the common biliary pattern.[20,24] On the other hand, 7α-hydroxycholesterol-^{14}C was metabolized in a markedly different pattern from the common one: first, radioactivity of the dihydroxy bile acid fraction was much higher than that observed when cholesterol-^{14}C was given (Figs. 5, A and B); second, the share of cholic acid in the trihydroxy bile acid fraction was rather small (40 %) (Table 5), while an appreciable amount of α-muricholic acid, a further oxidized metabolite of chenodeoxycholic acid, was detected therein.[20] These observations prompted the author to study further the metabolism of 7α-hydroxycholesterol-^{14}C *in vitro* as well as *in vivo*.

As described above (I. a. i.), the acidic fraction derived from 7α-hydroxycholesterol-^{14}C incubated with the mitochondrial fraction of rat liver homogenate showed a characteristic pattern of the metabolites: first, the trihydroxy bile acid fraction contained α-muricholic acid, but not cholic acid; second, the dihydroxy bile acid fraction was composed of chenodeoxycholic acid and the side-chain cleaved product of the starting material (Δ^5-acid).[10] These findings strongly indicate that, when 7α-hydroxycholesterol is metabolized in the mitochondria, its side-chain is cleaved first and the resulting Δ^5-acid is transformed into chenodeoxycholic acid, which, in turn, is further oxidized to α-muricholic acid.[21]

* Their respective metabolites may be included.

The similar experiment *in vitro* with the mitochondrial fraction of rabbit liver homogenate[11] also showed that 7α-hydroxycholesterol was metabolized to chenodeoxycholic acid and the Δ^5-acid, any trihydroxy bile acid being undetectable in the reaction mixture (I. a. ii).

Some years before these experiments were performed, Usui and Yamasaki (1964)[32] reported that, when the Δ^5-acid, which was then taken for a model compound of 7α-hydroxycholesterol, was given to bile fistula rats, it was effectively converted to chenodeoxycholic acid together with a muricholic acid fraction, which was later proved to contain α- and β-muricholic acids (II. c. i).

Based on these observations, the author and his associates (1971)[9] proposed the alternative biogenetic pathway of C_{24}-bile acids with special reference to chenodeoxycholic acid, where the Δ^5-acid is a key intermediate, as shown in Chart C.

Among the steps of the metabolic sequence concerned, the Δ^5-acid (a) was dehydrogenated to give 7α-hydroxy-3-oxochol-5-enoic aicd (3-oxo Δ^5-acid, b) (step a$\rightarrow$ b) by incubation with the microsomal fraction of rat liver homogenate fortified with NADP and it was readily converted by HCl-treatment to its isomer, 7α-hydroxy-3-oxochol-4-enoic acid (3-oxo Δ^4-acid, c).[12] This 3-oxo-Δ^4-acid(c) was effectively converted to chenodeoxycholic acid(e) together with α- and β-muricholic acids, when it was given to bile-fistula rats.[13] It was also converted readily to chenodeoxycholic acid by incubation with the supernatant fraction of rat liver homogenate fortified with NADPH.[13]

Although the steps, b $\rightarrow$ c ($\Delta^5 \rightarrow \Delta^4$-isomerase activity) and c $\rightarrow$ d (5β-reductase activity) have not yet been demonstrated, it might be certain that the metabolic sequence shown in Chart C does occur in the rat liver, because the Δ^5-acid was proved to be converted effectively to chenodeoxycholic acid in the experiment *in vivo*, as mentioned above.[32]

By chance, it was found that a Lifschütz-positive spot was detected on a thin-layer chromatogram of the dihydroxy bile acid fraction obtained from a hydrolyzed bile sample of bile-fistula rats. Ayaki and Yamasaki[28] demonstrated that, when cholesterol-^{14}C was given to bile-fistula rats, the radioactive Δ^5-acid was isolated from the bile and identified as such. At the same time Yamasaki and Yamasaki[29] showed that, when 7α-hydroxycholesterol-^{14}C was given to hens fitted with a bile-fistula, the Δ^5-acid(a) and its isomer, the Δ^4-acid(f), were isolated from the bile and identified as such. The finding, that the Δ^4-acid was derived from 7α-hydroxycholesterol, strongly indicates that 7α-hydroxy-3-oxochol-4-enoic acid(c) is a member of the metabolic sequence of the author's pathyway (Chart C).

Furthermore, the Δ^5-acid and the Δ^4-acid were isolated from human and

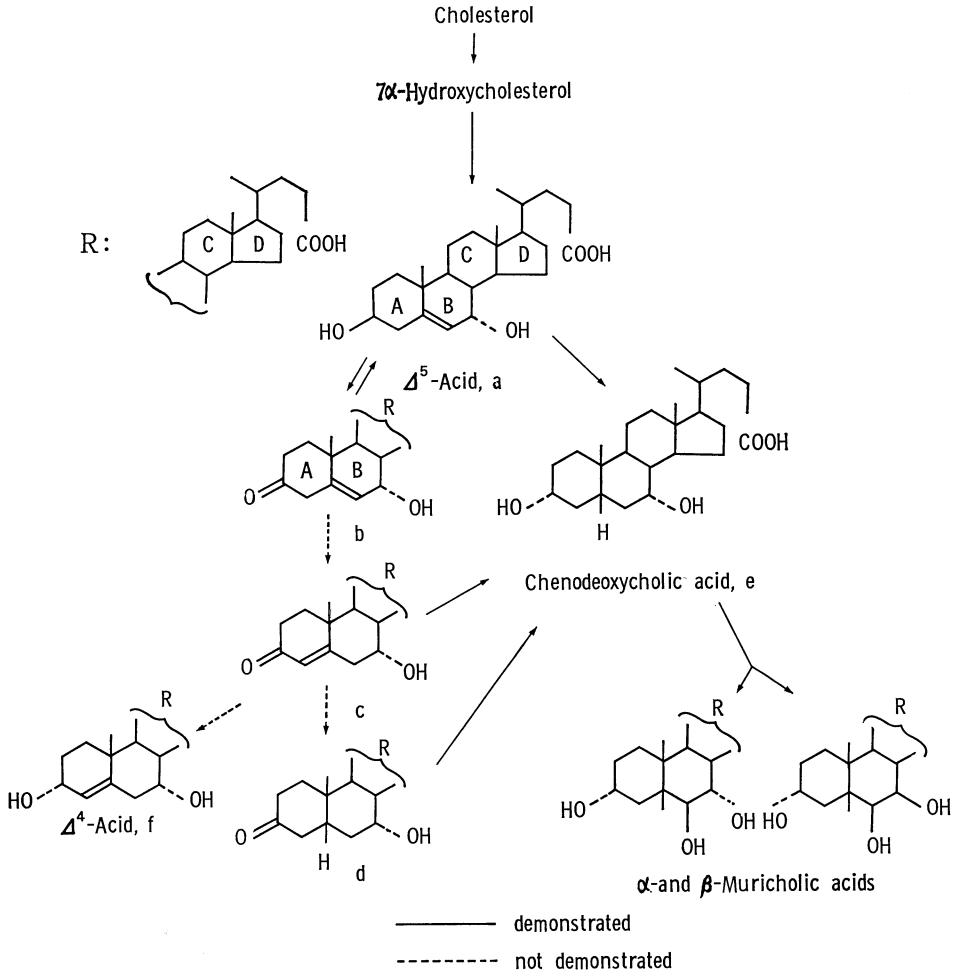

Chart C The alternative biogenetic pathway of chenodeoxycholic acid proposed by the author's group.

hen biles, even though their amounts were not high, and identified as such by means of GLC and MS or GC–MS[41,42] (III. a ; III. b). It must be emphasised here that these successful results were based on the observations mentioned above (IV. a. ii ; IV. b). Since these allylic bile acids are acid- and heat-labile, they must be carefully treated so as to minimize the possibility of attack by H^+ and methoxylated in methanol-H^+ to insure against heat decomposition on GLC, MS or GC–MS.

As a subsidiary evidence for the author's pathway, Yamaga[15,16] showed that the Δ^5-acid was converted not only to chenodeoxycholic acid but also to

cholic acid and allocholic acid, together with allochenodeoxycholic acid, when the Δ^5-acid-^{14}C was given intraperitoneally to carp, whose major bile salt is 5α-cyprinol sulfate. Hoshita[52] and Masui *et al.*[53] indicated by experiments *in vivo* and *in vitro*, that 5α-cyprinol was probably a primary metabolite in carp bile. These results suggested that the Δ^5-acid was converted not only to chenodeoxycholic acid and allochenodeoxycholic acid but also to cholic acid and allocholic acid in spite of the current view of cholic acid biogenesis, even after the side-chain of cholesterol or its C_{27}-derivatives was cleaved, in accordance with the observation that chenodeoxycholic acid was converted to cholic acid in the eel liver.[34] As for generalization of 12α-hydroxylation of any C_{24}-bile acid in the fish liver, however, further observation should be waited.

The metabolic studies of 7β-hydroxycholesterol[39] and its side-chain cleaved product, $3\beta, 7\beta$-dihydroxychol-5-enoic acid,[40] might provide another subsidiary evidence for the author's pathway, where either of these steroids proved to be scarcely converted to any natural bile acid.

Elucidation of the biochemical or clinical significance of the occurrence of the Δ^5-acid and the Δ^4-acid in bile should wait for further information. But some available data might suggest that there prevails the condition similar to that provided by the exogenous administration of 7α-hydrocholesterol: This hydroxycholesterol has been known for a long time to occur in animal tissues or organs besides the liver.[54] In 1961 Boyd and Mawer[22] isolated 3β-acyl esters of 7α-hydroxycholesterol from the liver and serum of rats. These esters of extrahepatic origin would be expected to be carried by the blood to the liver. If it were actually so, these esters were hydrolyzed like cholesterol esters (Sakamoto)[23] and preferentially taken up by the mitochondria of the liver cells and then they were metabolized in such a manner as 7α-hydroxycholesterol administered exogenously, as described above (II. a). And it would reasonably be expected that the Δ^5-acid together with its metabolic by-product, the Δ^4-acid, is excreted as a natural component of the bile.

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