The minor bile acids of the toad, *Bufo vulgaris formosus*[1]

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Abstract Bile from the toad, *Bufo vulgaris formosus,* was found to contain a number of minor bile acids along with two major bile acids, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid and 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid. The following minor bile acids were identified by combined gas–liquid chromatography–mass spectrometry: cholic acid, allocholic acid, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid, 3α,7α,12α-trihydroxy-5α-cholest-22-ene-24-carboxylic acid, 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid, varanic acid, and 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid. The fact that the toad bile contains not only cholic acid but also 3α,7α,12α-trihydroxy- and 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acids, which have been recognized as biosynthetic intermediates of cholic acid in mammals, suggests that the toad is capable of synthesizing cholic acid by the same pathway as that for the biosynthesis of the C_{24} bile acid in mammals.—**Une, M., T. Kuramoto, and T. Hoshita.** The minor bile acids of the toad, *Bufo vulgaris formosus. J. Lipid Res.* 1983. **24:** 1468–1474.

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The bile of the toad, *Bufo vulgaris formosus,* has been known to contain two unique higher bile acids, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid and 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (1, 2). The former is the only unsaturated C_{28} steroid to be included as a bile acid. Another special chemical feature of this bile acid is that the location of the carboxylic group of the C_{28} bile acid differs from that of the same group of all other bile acids so far identified as natural products. The latter of the two unique bile acids possesses a double bond in an unusual position. Furthermore, the biological origin of these unsaturated higher bile acids is obscure. Intraperitonal injection of the toad with [4-^{14}C]cholesterol as well as [2-^{14}C]mevalonate led to the formation of a number of radioactive bile acids and bile alcohols, but the two unsaturated higher bile acids did not become labeled (3, 4).

In order to obtain at least partial information about the biogenesis of bile acids in the toad, a more complete knowledge of the biliary components of the toad must be secured. We have now examined the minor bile acids in the bile of this species by means of a combination of gas–liquid chromatography and mass spectrometry.

MATERIALS AND METHODS

Gas–liquid chromatography–mass spectrometry

GLC–MS was carried out on a Shimadzu GC-MS-7000 gas chromatograph–mass spectrometer. The following operation conditions were employed: column, 3% OV-17 (1m × 3mm); column temperature, 280°C; ionizing current, 60 μA; ionizing voltage, 70 eV.

Reference bile acids

Cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) was a commercial product. Allocholic acid (3α,7α,12α-trihydroxy-5α-cholan-24-oic acid) (5), 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (6), 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (7), 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (2), and 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid (1), were isolated from natural sources. Varanic acid (3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid) (8) and 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid (9) were prepared in this laboratory according to the methods described previously.

Extraction and fractionation of unconjugated bile acid mixture from the toad bile

Bile was collected by putting the gallbladders of the toad into ethanol. Evaporation of the filtered solution left crude bile salts. The crude bile salts (8.5 g) were

Abbreviations: GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TLC, thin-layer chromatography; RRT, relative retention time; TMS, trimethylsilyl.

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dissolved in 200 ml of water and the solution was acidified with 2 N HCl and then extracted with three 150-ml portions of ether. The ethereal extracts were combined and washed with three 100-ml portions of 5% Na_2CO_3 solution to extract acidic materials. The Na_2CO_3 washings were combined, acidified with 2 N HCl, and re-extracted with three 150-ml portions of ether. The pooled ethereal extracts were washed with water, dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness, leaving a residue (1.4 g) consisting of unconjugated bile acids. The bile acid mixture was methylated by the usual manner with diazomethane and then chromatographed on a silica gel column (100 g) eluted with acetone–ethyl acetate mixtures. The column effluents were monitored by thin-layer chromatography (silica gel G plate; solvent system, ethyl acetate–acetone 7:3).

RESULTS

Since biliary bile acids of the toad have been known to exist as unconjugated forms (1–4), they were isolated by ethereal extraction of the acidified bile. Preliminary GLC–MS analysis of the ethereal extracts revealed a complex bile acid mixture. Thus, a group separation was undertaken to simplify the bile acid pattern and to purify the sample further. The bile acid mixture was methylated and chromatographed on a silica gel column to get five fractions (**Table 1**). **Fig. 1** shows the GLC analysis of bile acid methyl esters as the TMS ether derivatives after the group separation and demonstrates the presence of at least ten different bile acids which are tentatively named as bile acids I–X. Mass fragment ions of these bile acids as the methyl ester-TMS ether derivatives are shown in **Table 2** with their RRTs on GLC. The table further shows the relative abundance of the listed bile acids in the mixture as determined by GLC.

Bile acid I was identified as 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid. The methyl ester-TMS ether derivative had the same RRT and mass spectrum of the authentic compound. The spectrum shows the molecular ion at m/z 692, and a series of fragment ions at m/z 602, 512, and 422 which are formed by the successive loss of the TMS groups from the molecular ion. The base peak at m/z 253 and the peak at m/z 343 represent loss of the side chain plus three and two nuclear TMS groups, respectively. The fragment at m/z 315 is derived by cleavage of the 6,7- and 9,10-bonds along with the loss of the 7α- and 12α-TMS groups.

Bile acid II was identified as 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid by direct comparison of the RRT and mass spectrum with the authentic compound. The spectrum shows a weak molecular ion at m/z 680, a series of peaks at m/z 590 (M-90), 500 (M-2 × 90), and 410 (M-3 × 90), peaks at 665 (M-15), and 343 [M-(side chain + 2 × 90)], and the base peak at 253 [M-(side chain + 3 × 90)]. A weak ion was seen at m/z 303. This fragment is formed by cleavage of the 6,7- and 9,10-bonds plus the loss of 7α- and 12α-TMS groups.

Bile acid III was identified as 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid. The RRT and mass spectrum of the methyl ester-TMS ether derivative of bile acid III were identical with those of the corresponding derivative of the authentic compound. In the mass spectrum, the molecular ion was absent, but the peak due to the loss of a methyl group from the molecular ion was seen at m/z 663. There were two series of fragments, one at m/z 588, 498, and 408, and a second at m/z 461, 371, and 281. The former series results from the successive loss of one, two, and three molecules of TMS-OH from the molecular ion. The latter series results from scission of the bond between C-20 and C-22 followed by the successive loss of TMS-OH molecules. Generally, the base peak of the methyl ester-TMS ether derivatives of bile acids carrying the cholic acid type nucleus appears at m/z 253, [M-(side chain + 3 × 90)]. Although this spectrum shows a peak at m/z 253, the base peak was seen at m/z 281. These observations suggest a facile rupture of the bond between C-20 and C-22 in bile acid III which has the Δ^{23} double bond.

The ratio of the RRTs between bile acid IV and bile acid I was in good agreement with the constant separating factor found between the pairs of bile acids car-

TABLE 1. Silica gel column chromatography of bile acids from the toad as methyl esters

Fraction	Eluant	Vol.	Wt.	Eluate
		ml	*mg*	
A	Ethyl acetate	500	937	Trihydroxy-5β-higher bile acids (I, II, III)
B	10% Acetone-ethyl acetate	200	58	Trihydroxy-5α-higher bile acids (IV, V, VI)
C	12% Acetone-ethyl acetate	200	52	Cholic acid (VII, major) and allocholic acid (VIII)
D	20% Acetone-ethyl acetate	200	8	Cholic acid (VII) and allocholic acid (VIII, major)
E	25% Acetone-ethyl acetate	200	35	Tetrahydroxy-bile acids (IX, X)

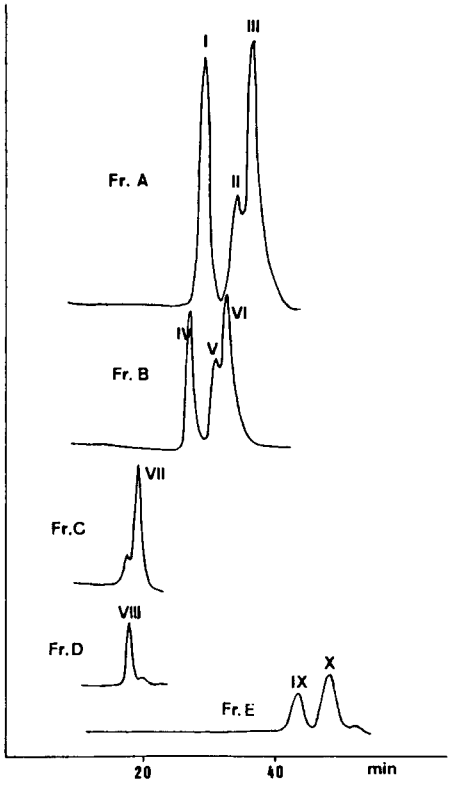


Fig. 1. Gas–liquid chromatographic separation of bile acid methyl esters as TMS ether derivatives of each fraction after silica gel column chromatography.

rying the allocholic acid type nucleus and their 5β-counterparts under the employed condition. The mass fragmentation pattern of the methyl ester-TMS ether derivative of bile acid IV was very similar to that of the corresponding derivative of bile acid I, except for a difference of the intensities of the fragments at m/z 315 and 343. The intensities of the fragments at m/z 315 (37%) and 343 (49%) in the spectrum of bile acid IV were much greater than those [m/z 315, (7%), m/z 343, (22%)] in that of bile acid I. These results strongly suggest, but do not prove, that bile acid IV is the 5α-isomer of bile acid I, namely, 3α,7α,12α-trihydroxy-5α-cholest-22-ene-24-carboxylic acid.

Bile acid V was identified with authentic 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid by direct comparison of the RRT and mass spectrum. When this spectrum was compared to one obtained from bile acid II, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid, the intensities of the fragment ions at m/z 500, (M-2 $\times$ 90) and 343 [M-(side chain + 2 $\times$ 90)] were greater than the ions at m/z 410 (M-3 $\times$ 90) and 253 [M-(side chain + 3 $\times$ 90)], respectively, for the 5α-bile acid, while the reverse was true for the 5β-bile acid. Furthermore, the intensity of the fragment at m/z 303 of bile acid V was much greater than that of bile acid II.

The mass spectrum of the methyl ester-TMS ether derivative of bile acid VI was almost identical to that obtained from the corresponding derivative of bile acid III. A difference between the two spectra was seen, however, in the relative intensities of some peaks. The intensities of the peaks at m/z 371 and 343 were greater than the peaks at m/z 281 and 253, respectively, for bile acid VI, while the reverse was true for bile acid III. The mass fragment ion at m/z 301 was seen in both spectra of the methyl ester-TMS ether derivatives of bile acids VI and III. The intensity of the ion formed from the former was greater than that from the latter. The mass spectral evidence together with the observation that the ratio of the RRTs of the methyl ester-TMS ether derivatives of bile acids VI and III was equal to the RRT ratio of the corresponding derivatives of 5α- and 5β-bile acids suggests, but does not prove, that bile acid VI is the 5α-isomer of bile acid III, namely, 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid.

Bile acids VII and VIII were identified as cholic acid and allocholic acid, respectively. The RRTs and mass spectra of the methyl ester-TMS ether derivatives of bile acids VII and VIII were completely identical with those of the TMS ether derivatives of authentic methyl cholate and methyl allocholate, respectively.

Bile acid IX was identified as varanic acid. The methyl ester-TMS ether derivative of bile acid IX had the same RRT and mass spectrum as the authentic compound. The spectrum shows the base peak at m/z 253 and major peaks at m/z 588, 498, 343, and 281. The peak at m/z 321 is attributed to cleavage between C-24 and C-25 followed by elimination of four TMS-OH molecules.

Bile acid X was identified as 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid by direct comparison of the RRT and mass spectrum with the authentic compound. The mass spectrum of the methyl ester-TMS ether of bile acid X was closely similar to that of the corresponding derivative of varanic acid (IX) with respect to peak intensities and fragmentation patterns. The difference was that the peaks in the spectrum of bile acid X were shifted 14 mass units upfield because of the additional methyl group present at C-24. Another difference was that in the spectrum of bile acid X, the base peak appears at m/z 203. This is a side chain fragment resulting from the scission of the bond between C-23 and C-24.

DISCUSSION

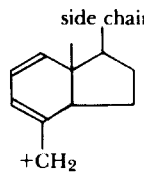
Until the present study, only two major bile acids, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid (I) and 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (III) had been isolated from toad bile. The present

TABLE 2. Relative abundances of important fragment ions of bile acids of the toad as methyl ester–TMS ethers[a]

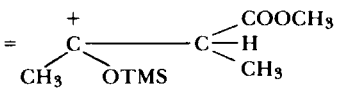
Fragment Ion	m/z	I	IV	m/z	II	V	m/z	III	VI	m/z	VII	VIII	m/z	IX	m/z	X
[M]	692	6	10	680	1	1	678		4	638			768		782	
[M-15]	677			665	7	4	663	3		623	2	2	753	7	767	9
[M-90]	602	29	49	590	3	4	588	7	21	548			678		692	4
[M-(90 × 2)]	512	23	27	500	65	100	498	12	22	458	10	2	588	33	602	31
[M-(C_{22}–C_{27} + 90)]				461			461	6	39							
[M-(90 × 3)]	422	28	7	410	85	1	408	13	11	368	96	26	498	67	512	25
[M-(90 × 4)]													408	10	422	14
[M-(C_{22}–C_{27} + 90 × 2)]				371			371	26	100							
[M-(side chain + 90 × 2)]	343	22	49	343	34	77	343	13	55	343	34	100	343	42	343	22
[M-(C_{25}–C_{27} + 90 × 4)]													321	15		
Fragment A[b]	315	7	37	303	1	30	301	7	15	261		28				
[M-(C_{22}–C_{27} + 90 × 3)]				281	12	5	281	100	79				281	27	281	11
[M-(side chain + 90 × 3)]	253	100	100	253	100	25	253	43	46	253	100	64	253	100	253	17
Fragment B[c]															203	100
% of total		42.0	2.4		11.6	0.8		32.5	1.9		4.1	0.9		2.0		1.8
RRT[d] on GLC[e]		1.40	1.24		1.63	1.43		1.72	1.53		1.00	0.88		2.17		2.52
Ratio of 5α/5β		0.89			0.88			0.89			0.88					

[a] Bile acids I–X were identified as follows: I, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid; II, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; III, 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid; IV, 3α,7α,12α-trihydroxy-5α-cholest-22-ene-24-carboxylic acid; V, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid; VI, 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid; VII, cholic acid; VIII, allocholic acid; IX, varanic acid; X, 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid.

[b] Fragment A =



[c] Fragment B =



[d] Relative to methyl cholate-TMS ether.
[e] Column, 3% OV-17; column temperature, 280°C.

study demonstrates the presence of the following minor bile acids in toad bile: 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (II); 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (V); 3α,7α,12α-trihydroxy-5α-cholest-22-ene-24-carboxylic acid (IV); 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid (VI); cholic acid (VII); allocholic acid (VIII); varanic acid (IX); 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid (X). The two unsaturated 5α-bile acids (IV and VI) were not fully identified because of the lack of standards. It is well known that mass spectra of isomeric 5α- and 5β-bile acids are similar, i.e., the process of fragmentation is essentially the same (10). Only occasional differences are discerned in the relative intensities of selected fragment ions. Elliott (11) has reported a more facile elimination of the 3α-TMS group in 5β-bile acids than in their 5α-isomers. The intensity of the fragment at m/z 343 [M-(side chain + 2 × 90)] is greater in the latter than in the former. It has been reported that the intensity of the fragment ion resulting from cleavage of the 6,7- and 9,10-bonds in the spectra of the methyl ester-TMS ether derivatives of 5α-bile acids is greater than in the comparable 5β-bile acids (11). Based on this evidence, tentative identifications of IV and VI were made not only by the comparisons of their gas–liquid chromatographic retention time ratios but also by the comparison of their mass spectra with the two major 5β-bile acids, I and III, respectively. The remainder of the minor acids were identified with certainty by direct comparison of their chromatographic properties and mass spectra to those of authentic compounds.

The presence of cholic acid (VII) in toad bile had been postulated since labeled cholic acid was obtained from the toad that received [2-^{14}C]mevalonate (4). The study described here confirmed the presence of this C_{24} bile acid (VII). The biosynthetic sequence between cholesterol and cholic acid in mammals has been extensively studied (12). At the present time, it is believed that the major pathway for the mammalian cholic acid biosynthesis involves the following intermediates (**Fig. 2**): cholesterol (XI) → cholest-5-ene-3β,7α-diol (XII) → 7α-hydroxycholest-4-en-3-one (XIII) → 7α,12α-dihydroxycholest-4-en-3-one (XIV) → 5β-cholestane-3α,7α,12α-triol (XV) → 5β-cholestane-3α,7α,12α,26-tetrol (XVI) → 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (II) → varanic acid (IX) → cholic acid (VII). The presence and formation from cholesterol of the tetrol (XVI) in the toad has been reported by Hoshita et al. (3). The demonstration of the previous and present studies that the intermediates, 5β-cholestanetetrol (XVI), trihydroxy-5β-cholestanoic acid (II), and varanic acid (IX), occur in toad bile strongly suggest that the pathway for the synthesis of cholic acid (VII) in the toad is the same as that in mammals.

The present study also demonstrates the presence of another C_{24} bile acid which was identified as the 5α-isomer of cholic acid, allocholic acid (VIII). Current information suggests that allocholic acid is derived from either cholestanol or cholesterol. Cholestanol has been shown to be converted to allocholic acid in rats (13) and gerbils (14) by a pathway similar to that for the biosynthesis of cholic acid from cholesterol. However, it is hardly conceivable that the major source of the 5α-bile acid (VIII) found in toad is cholestanol, since very little

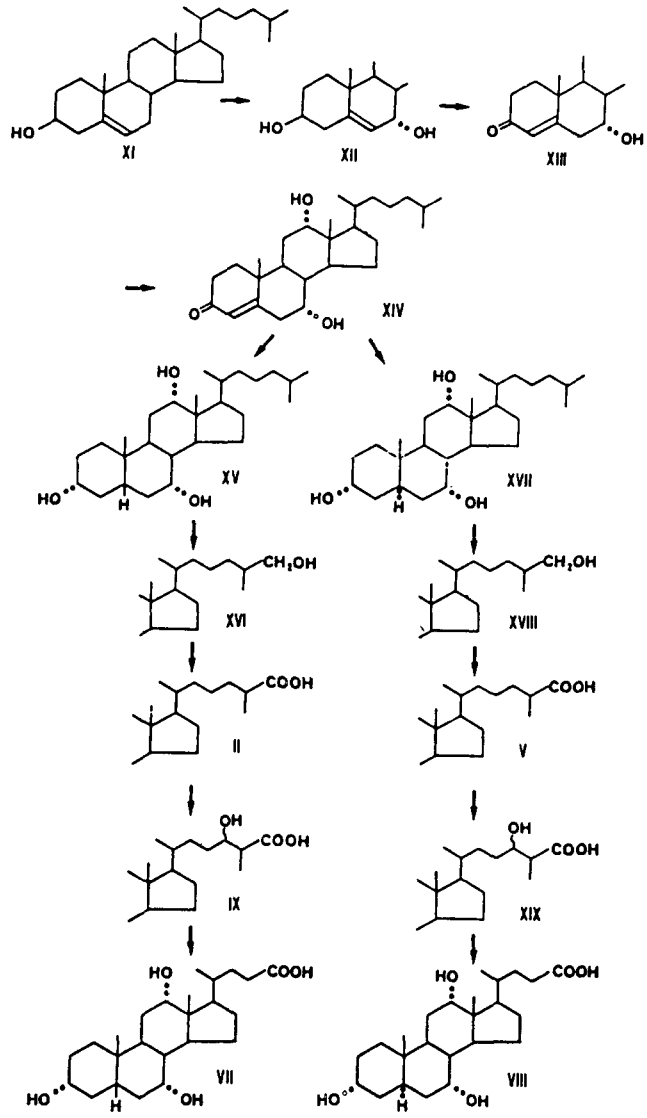


Fig. 2. Biosynthetic route of cholic acid and allocholic acid. II, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; V, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid; VII, cholic acid; VIII, allocholic acid; IX, varanic acid; XI, cholesterol; XII, cholest-5-ene-3β,7α-diol; XIII, 7α-hydroxycholest-4-en-3-one; XIV, 7α,12α-dihydroxycholest-4-en-3-one; XV, 5β-cholestane-3α,7α,12α-triol; XVI, 5β-cholestane-3α,7α,12α,26-tetrol; XVII, 5α-cholestane-3α,7α,12α-triol; XVIII, 5α-cholestane-3α,7α,12α,26-tetrol; XIX, 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid.

of this 5α-sterol was found in toad liver (our unpublished observation). Hoshita, Shefer, and Mosbach (15) have shown that liver microsomes from the green iguana, a species in which the major biliary bile acid is the taurine conjugate of allocholic acid, convert 7α,12α-dihydroxycholest-4-en-3-one (XIV) into 5α-cholestane-3α,7α,12α-triol (XVII) rather than the 5β-isomer (XV) involved in cholic acid biosynthesis. This indicates that allocholic acid can be formed from cholesterol by a modification of the biosynthetic pathway to cholic acid in which the only difference is the stereospecific saturation of Δ^4-double bond of XIV. Thus, 7α,12α-dihydroxycholest-4-en-3-one (XIV) is the last intermediate common to both cholic acid (VII) and allocholic acid (VIII). 5α-Cholestane-3α,7α,12α,26-tetrol (XVIII) and 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid (V) seem to be the intermediates in the pathway of biosynthesis of allocholic acid (VIII) from the 5α-cholestanetriol (XVII). Hoshita et al. (3) have reported the occurrence and formation from cholesterol of the 5α-tetrol (XVIII) in the toad, and the present study demonstrates the presence of the trihydroxy-5α-cholestanoic acid (V) in toad bile. These facts suggest that in the toad a lesser amount of 7α,12α-dihydroxycholest-4-en-3-one (XIV), a greater fraction of which is converted to 5β-cholestane-3α,7α,12α-triol (XV), is transformed into 5α-cholestane-3α,7α,12α-triol (XVII) and then into the 5α-C_{27}-tetrol (XVIII), the 5α-C_{27}-bile acid (V), and allocholic acid (VIII).

The present study demonstrates the presence in a lesser amount of the saturated 5β-C_{28}-bile acid, 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid (X). The structure of this tetrahydroxy-C_{28}-bile acid (X) closely resembles that of varanic acid (IX). The only difference between X and IX is the existence or the absence of the C-28 methyl group. As a small amount of 24-methylcholesterol, presumably campesterol (XX), has been found in toad liver (16), it is likely that the C_{28} bile acid (X) is formed from the C_{28} sterol (XX) by a pathway similar to that for the biosynthesis of the C_{27} bile acid (IX) from cholesterol. In contrast to IX, a part of which is further oxidized to cholic acid (VII) with the intermediary formation of the β-keto acid, the 24-methylated bile acid (X) would not be further oxidized since the 24-hydroxyl group is tertiary and could not be oxidized to a keto group.

The present finding of 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid (X) suggests a biochemical origin of the most abundant toad biliary bile acid, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid (I). Although the position of the carboxyl group of the major C_{28} bile acid (I) differed from that of the same group of the minor C_{28} bile acid (X), the side chain carbon skeletons of these bile acids are identical to each other and to that of campesterol (XX). Since the oxidation of the C-26 methyl group of campesterol (XX) would produce the carboxyl group of X, whereas the oxidation at C-28 methyl group would lead to the carboxyl group of I, it is not unreasonable to assume that the C_{28} sterol (XX) is the source common to both the C_{28} bile acids, I and X (**Fig. 3**).

3α,7α,12α - Trihydroxy - 5α - cholest - 22 - ene - 24 - carboxylic acid (IV) seems to be formed from campesterol (XX) by a modification of the pathway to the 5β-isomer (I).

Recently, Ali, Stephenson, and Elliott (17) reported the presence of 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (III) in bile of *Varanus monitor*, the major bile acid of which is varanic acid (IX). The co-existence and the structural relationship of these C_{27} bile acids suggest that in this reptile the Δ^{23}-C_{27} bile acid, III, is a biological dehydration product of varanic acid (IX). However, it seems unlikely that in the toad, the second principal bile acid, III, is a metabolite of varanic acid (IX), because in our previous investigations (3, 4) with labeled cholesterol as well as mevalonate, the label was incorporated into cholic acid and its biosynthetic precursors in the toad, but the two major bile acids, III and I, did not become labeled. Until other evidence

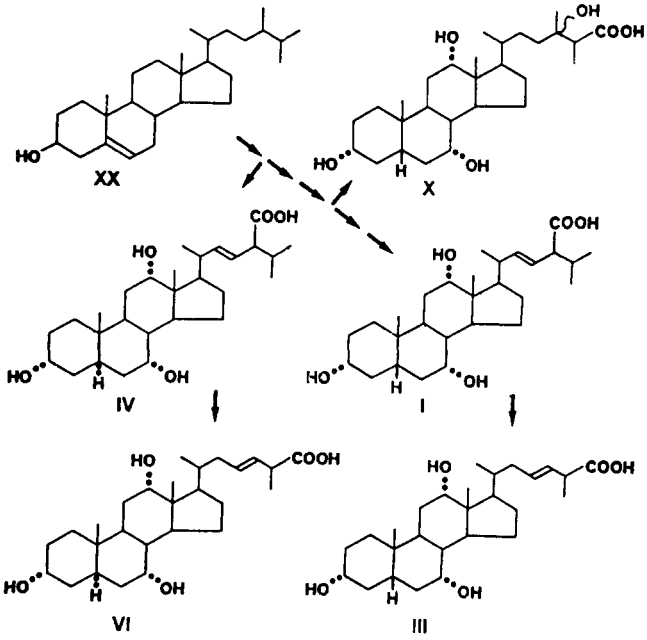


Fig. 3. Postulated biosynthetic route of higher bile acids in the toad. I, 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid; III, 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid; IV, 3α,7α,12α-trihydroxy-5α-cholest-22-ene-24-carboxylic acid; VI, 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid; X, 3α,7α,12α,24-tetrahydroxy-24-methyl-5β-cholestan-26-oic acid; XX, campesterol.

becomes available, we postulate that 3α,7α,12α-trihydroxy-5β-cholest-23-en-26-oic acid (III) is a metabolite of 3α,7α,12α-trihydroxy-5β-cholest-22-ene-24-carboxylic acid (I), because decarboxylation and migration of the double bond followed by oxidation of the terminal methyl group of the latter (I) would lead to the former (III). If this is correct, 3α,7α,12α-trihydroxy-5α-cholest-23-en-26-oic acid (VI) would be formed from the unsaturated 5α-C_{28}-bile acid (IV) by a pathway analogous to that for the formation of the 5β-isomer (III).

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