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Bile Salts of Anura

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Bile salts of six different species of the Anura were investigated by means of thin-layer chromatography, gas–liquid chromatography, and mass spectrometry. Bile of *Xenopus laevis* contained taurine-conjugated and free forms of 3α,7α,12α-trihydroxy-5β-cholestanoic acid and 5β-cyprinol sulfate[2] as its chief constituents. Smaller amounts of cholic acid and chenodeoxycholic acid were also detected in the hydrolyzed bile. Neither conjugated bile acids nor bile alcohol sulfates were found in the bile of *Bombina orientalis*, the chief constituents of which were free 3α,7α,12α-trihydroxy- and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acids. *Hyla arborea japonica* contained 5β-ranol sulfate as almost the only bile salt. *Rana brevipoda* contained taurine-conjugated and free forms of cholic acid and 3α,7α,12α-trihydroxy-5β-cholestanoic acid, and 5β-cyprinol sulfate. *Rana rugosa* had mainly 5α-cyprinol sulfate in its bile. There was also a minor amount of tauro-3α,7α,12α-trihydroxy-5α-cholestanoate. *Rana limnocharis limnocharis* contained taurine-conjugated and free forms of cholic acid and 3α,7α,12α-trihydroxy-5β-cholestanoic acid, and 5β-bufol sulfate.

During the course of comparative studies on the bile salts, only seven species of amphibians have been investigated, but with remarkable results.[3] All the amphibians examined have unusual bile salts as their chief bile constituents, and the bile salt patterns of amphibians are complicated and differed from one another. This character is taxonomically useful and may throw light on molecular evolution of bile salts. It may be worthwhile in this situation to continue much more chemical work on bile salts of amphibians.

In this paper, we report examinations on the bile salts of the following Anura: *Xenopus laevis, Bombina orientalis, Hyla arborea japonica, Rana brevipoda, Rana rugosa,* and *Rana limnocharis limnocharis*.

Material and Method

1. **Extraction and Hydrolysis of Bile Salts**——Gall-bladders collected were cut under EtOH in a Waring-blender, and the EtOH extract was filtered. Evaporation of the solvent from the filtrate left crude bile salts as a solid. The bile salts were acetylated and dissolved in a 40% (w/v) solution of trichloroacetic acid in dioxan according to the procedure reported by Haslewood and Tökés.[4] After 2 weeks, the reaction mixture was diluted with H_2O and extracted with EtOAc. The upper phase (EtOAc extract) was washed with 0.5N NaOH, and then with H_2O. The lower aqueous layer and the washings were combined and concentrated to dryness. The residue was dissolved repeatedly in EtOH and filtered to remove undissolved inorganic materials. Evaporation of the solvent from the filtrate left a gum. The gum was heated with 2.5N KOH in a sealed metal container at 160° for 8 hr. The hydrolyzate was acidified with dil. HCl and extracted with ether. Evaporation of the solvent from the extract left an acidic residue ("acids").

The washed EtOAc layer which contained a mixture of neutral compounds including partially acetylated bile alcohols was concentrated to dryness. A solution of the residue in 2.5N MeOH–KOH was refluxed

1) Location: *Kasumi 1-2-3, Hiroshima.*
2) Systematic names of bile acids and bile alcohols refered to in the text by their trival names are listed in Table I.
3) G.A.D. Haslewood, *J. Lipid Res.*, **8**, 535 (1967).
4) G.A.D. Haslewood and L. Tökés, *Biochem. J.*, **126**, 1161 (1972).

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on a water bath for 2 hr. The hydrolyzate was diluted with H_2O and extracted with a mixture of EtOAc and *n*-BuOH (1: 1, v/v). Evaporation of the solvents left a neutral residue ("neutrals").

2. Chromatography——Thin-layer chromatography (TLC) of bile salts was performed according to the procedures reported by Sasaki[5] by using a Silica gel G plate (20×20 cm) in a layer of 250 μ, with *n*-BuOH–AcOH–H_2O (17: 2: 1, v/v) and $CHCl_3$–MeOH–AcOH–H_2O (13: 4: 2: 1, v/v) as the moving phases for two-dimensional development. The spots were detected by spraying 10% phosphomolibdic acid in ethanol. A typical separation of bile salts by this method is shown in Fig. 1.

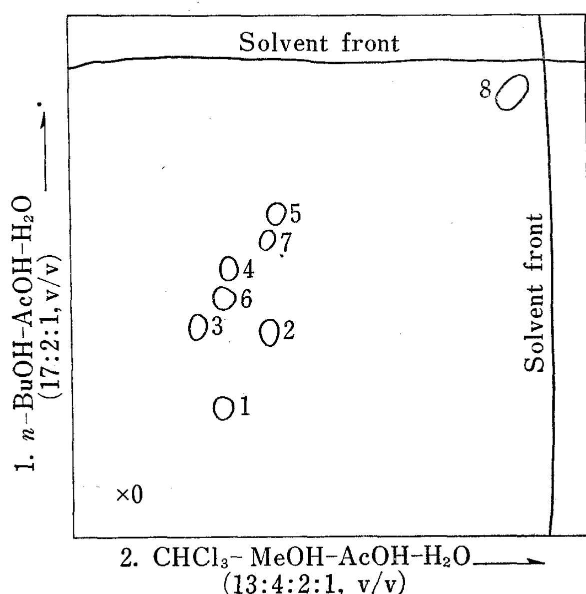

Fig. 1. Separation of Bile Salts by Two-Dimensional Development Thin-Layer Chromatography

0: origin, 1: taurotrihydroxycholanoates, 2: taurotrihydroxycholestanoates, 3: cholestanehexol sulfates, 4: cholestanepentol sulfates, 5: cholestanetetrol sulfates, 6: norcholestanepentol sulfates, 7: norcholestanetetrol sulfates, 8: free bile acids

TLC of free bile acids, their methyl esters, and free bile alcohols was performed as previously described[6] by using a 250 μ thick layer of Silica gel G with the following solvent systems: S-7, benzene–Me_2CHOH–AcOH (30:10: 1, v/v); EA-2, EtOAc–acetone (7: 3, v/v); CE-2, $CHCl_3$–EtOH (4: 1, v/v); EAW-2, EtOAc–AcOH–H_2O (17:2:1, v/v). For preparative TLC, a 1.25 mm thick layer of the absorbent was used. The steroid zones were detected by exposure of the plate to iodine vapor.

Gas–liquid chromatography (GLC) was performed with a Shimadzu gas chromatograph Model GC-4B equipped with a hydrogen flame ionization detector. The column was a glass tube, 2 m×4 mm, packed with 1% QF-1, 1.5% OV-1, or 1% OV-17 on 100—120 mesh Gas Chrom-Q (Applied Science Laboratories, Inc., U.S.A.). Approximate column conditions were; column temperature, 240° or 250°; detector temperature, 280°; carrier gas, nitrogen; carrier gas flow, 50 ml/min.

Methyl esters, trifluoroacetates, and trimethylsilyl ethers of the sample to be analyzed were prepared by using methods previously reported.[7]

3. Mass Spectral Measurements——The spectra were measured by the direct sample introduction technique with a Hitachi mass spectrometer Model RMS-4 under conditions of ion source, 160° and ionization voltage, 80 eV.

4. Reference Compounds——All the compounds listed in Table I were isolated from natural sources or were synthesized according to the procedures previously reported,[8] except as noted below.

Cholic acid and chenodeoxycholic acid were commercial products. 12-Deoxy-5α-cyprinol was synthesized from 5α-cyprinol tetraacetate[9] by oxidation with CrO_3 to give the 12-oxoderivative, followed by

5) T. Sasaki, *Hiroshima J. Med. Sci.*, **14**, 85 (1965).
6) *a*) P. Eneroth, *J. Lipid Res.*, **4**, 11 (1963); *b*) T. Kazuno and T. Hoshita, *Steroids*, **3**, 55 (1964).
7) *a*) J. Sjövall, *Acta Chem. Scand.*, **16**, 1761 (1962); *b*) D.H. Sandberg, J. Sjövall, and K. Sjövall, *J. Lipid Res.*, **6**, 182 (1965).
8) *a*) K. Amimoto, *J. Biochem.* (Tokyo), **59**, 340 (1966); *b*) K. Okuda, M.G. Horning, and E.C. Horning, *ibid.*, **71**, 885 (1972); *c*) T. Komatsubara, *Proc. Japan Acad.*, **30**, 614 (1954); *d*) S. Hayakawa, *ibid.*, **29**, 279 (1953); *e*) T. Hoshita, *J. Biochem.* (Tokyo), **46**, 507 (1959); *f*) T. Hoshita, K. Okuda, and T. Kazuno, *ibid.*, **61**, 756 (1967); *g*) T. Hoshita, T. Sasaki, Y. Tanaka, S. Betsuki, and T. Kazuno, *ibid.*, **57**, 751 (1965); *h*) Y. Inai, Y. Tanaka, S. Betsuki, and T. Kazuno, *ibid.*, **56**, 591 (1964); *i*) T. Hoshita, S. Shefer, and E.H. Mosbach, *J. Lipid Res.*, **9**, 237 (1968); *j*) T. Kazuno and A. Mori, *Proc. Japan Acad.*, **30**, 486 (1954); *k*) T. Hoshita, *J. Biochem.* (Tokyo), **52**, 125 (1962); *l*) K. Shimizu, F. Noda, and K. Yamasaki, *ibid.*, **45**, 625 (1956); *m*) W.H. Pearlman, *J. Am. Chem. Soc.*, **69**, 1475 (1947); *n*) T. Kazuno, T. Masui, and T. Hoshita, *J. Biochem.* (Tokyo), **50**, 12 (1962); *o*) T. Kazuno, T. Masui, and K. Okuda, *ibid.*, **57**, 75 (1965); *p*) A.D. Cross, *J. Chem. Soc.*, **1961**, 2817; *q*) T. Hoshita, S. Hirofuji, T. Nakagawa, and T. Kazuno, *J. Biochem.* (Tokyo), **62**, 62 (1967); *r*) T. Hoshita, *ibid.*, **52**, 176 (1962); *s*) T. Hoshita, S. Nagayoshi, and T. Kazuno, *ibid.*, **54**, 369 (1963); *t*) T. Hoshita, M. Kouchi, and T. Kazuno, *ibid.*, **53**, 291 (1963); *u*) R.J. Bridgwater, T. Briggs, and G.A.D. Haslewood, *Biochem. J.*, **82**, 285 (1962).
9) I.G. Anderson, T. Briggs, and G.A.D. Haslewood, *Biochem. J.*, **90**, 303 (1964).

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Huang-Minlon reduction. 26-Deoxy-5β-ranol was prepared from 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one[10] by reduction with $NaBH_4$. 5β-Cholane-3α,7α,12α,24-tetrol was prepared from methyl cholate by reduction with $LiAlH_4$.

Rf values on TLC and relative retention times on GLC of reference compounds are listed in Table I.

Table I. *Rf* values on TLC and Relative Retention Times on GLC of Bile Acids and Bile Alcohols

Bile acids and bile alcohols (trival names)	*Rf* values on TLC				Relative retention times[a)] on GLC		
	S-7	EA-2	CE-2	EAW-2	QF-1	OV-1	OV-17
Derivatives[b)]		Me			Me, TFA	Me, TMS	Me, TMS
3α,7α-Dihydroxy-5β-cholanoic acid (chenodeoxycholic acid)	0.69	0.91			0.69	0.96	1.11
3α,7α,12α-Trihydroxy-5α-cholanoic acid (allocholic acid)[8a)]	0.44	0.42			1.07	0.94	0.88
3α,7α,12α-Trihydroxy-5β-cholanoic acid (cholic acid)	0.51	0.54			1.00	1.00	1.00
3α,7α,12α-Trihydroxy-5α-cholestanoic acid[8b)]	0.53	0.63			1.65	1.60	1.43
3α,7α,12α-Trihydroxy-5β,25α-cholestanoic acid[8c)]	0.60	0.67			1.51	1.71	1.62
3α,7α,12α-Trihydroxy-5β,25β-cholestanoic acid[8c)]	0.60	0.67			1.51	1.71	1.62
3α,7α,12α-Trihydroxy-5β,25α-cholest-23-enoic acid[8d)]	0.60	0.66			1.51	1.69	1.71
3α,7α,12α-Trihydroxy-24-methyl-5β-cholestanoic acid[8e)]	0.61	0.73			1.67	1.93	1.84
3α,7α,12α-Trihydroxy-5β-cholestane-24-carboxylic acid[8f)]	0.57	0.77			1.42	1.67	1.57
3α,7α,12α-Trihydroxy-5β-cholest-22-ene-24-carboxylic acid[8g)]	0.59	0.74			1.28	1.45	1.41
3α,7α,12α,24-Tetrahydroxy-5β-cholestanoic acid[8h)]	0.28	0.46			2.29	2.48	2.16
5α-Cholestane-3α,7α,12α-triol[8i)]			0.78	0.92	0.59	0.77	0.56
5β-Cholestane-3α,7α,12α-triol[8j)]			0.78	0.92	0.54	0.82	0.62
5β-Cholane-3α,7α,12α,24-tetrol			0.46	0.57	1.02	1.05	0.75
27-Nor-5β-cholestane-3α,7α,12α,24-tetrol (26-deoxy-5β-ranol)			0.55	0.72	1.46	1.22	0.88
5α-Cholestane-3α,7α,26,27-tetrol (12-deoxy-5α-cyprinol)			0.62	0.72	2.12	2.94	2.13
5α-Cholestane-3α,7α,12α,26-tetrol[8k)]			0.62	0.73	1.58	1.64	1.10
5β-Cholestane-3α,7α,12α,24-tetrol[8l)]			0.65	0.79	1.12	1.37	0.98
5β-Cholestane-3α,7α,12α,25-tetrol[8m)]			0.62	0.74	—[c)]	1.55	1.09
5β-Cholestane-3α,7α,12α,26-tetrol[8n)]			0.64	0.76	1.46	1.74	1.25
27-Nor-5β-cholestane-3α,7α,12α,24,26-pentol (5β-ranol)[8o)]			0.19	0.36	2.46	2.28	1.57
5β-Cholestane-3α,7α,12α,24,26-pentol[8p)]			0.25	0.43	2.38	2.40	1.60
5α-Cholestane-3α,7α,12α,25,26-pentol (5α-bufol)[8q)]			0.26	0.42	—[c)]	2.54	1.55
5β-Cholestane-3α,7α,12α,25,26-pentol (5β-bufol)[8r)]			0.30	0.46	—[c)]	2.68	1.71
5α-Cholestane-3α,7α,12α,26,27-pentol (5α-cyprinol)[8s)]			0.26	0.42	3.56	2.83	1.82
5β-Cholestane-3α,7α,12α,26,27-pentol (5β-cyprinol)[8t)]			0.31	0.48	3.32	2.99	2.01
5β-Cholestane-3α,7α,12α,24,26,27-hexol (scymnol)[8u)]			0.11	0.23	4.04	3.37	2.06

a) Relative to trifluoroacetate (QF-1) or trimethylsilyl ether (OV-1, OV-17) of methyl cholate.
b) Me: methyl esters; TFA: trifluoroacetates; TMS: trimethylsilyl ethers
c) The trifluoroacetates of these compounds give several peaks, which may be due to degradation products.

10) T. Kazuno, A. Mori, K. Sasaki, and M. Mizuguchi, *Proc. Japan Acad.*, **28**, 421 (1952).

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Result and Discussion

1. Bile Salts of *Xenopus laevis*

Five gall-bladders of this species of Anura gave 27.9 mg of crude bile salts. TLC of the bile salts showed the presence of materials with the mobilities corresponding to taurotrihydroxycholestanoates, cholestanepentol sulfates, and free bile acids. Hydrolysis of the bile salts gave "neutrals" (20.1 mg) and "acids" (3.0 mg).

On TLC in the solvent system CE-2 or EAW-2, the "neutrals" gave a single spot which had the *Rf* value of 5β-cyprinol. The "neutrals" were subjected to preparative TLC in the solvent system CE-2, and the zone corresponding to 5β-cyprinol was eluted with ethanol. Evaporation of the solvent from the eluate left a gum. The gas chromatogram of the gum showed only one peak which had the same relative retention time as the trifluoroacetate or trimethylsilyl ether of 5β-cyprinol. The mass spectrum of the gum showed the same fragmentation pattern (peaks: *m/e* 434, 417, 416, 399, 387, 344, 299, 291, 289, 271, and 253) as that of authentic 5β-cyprinol.

On TLC in the solvent system S-7, the "acids" gave three spots, one corresponding to the mobility of 3α,7α,12α-trihydroxy-5β-cholestanoic acid, and the others moved at the same rates as cholic acid and chenodeoxycholic acid. After methylation and trifluoroacetylation, the same acids were evident on GLC (Fig. 2). Peaks 1, 2, and 3 had the same relative retention times as the trifluoroacetate-methyl esters of chenodeoxycholic acid, cholic acid, and 3α,7α,12α-trihydroxy-5β-cholestanoic acid, respectively.

The identity of these bile acids was further established by mass spectrometry. The methylated "acids" were separated into three components by preparative TLC in the solvent system EA-2. The first component was obtained from the area chromatographically corresponding to methyl chenodeoxycholate. The mass spectrum of this compound revealed the peaks at *m/e* 388, 370, 355, 273, 264, and 255. The mass numbers and the relative intensities of these peaks were identical with those in the spectrum of authentic methyl chenodeoxycholate. The mass spectrum of the second component, which ran at the same rate as methyl 3α,7α,12α-trihydroxy-5β-cholestanoate on the silica gel plate, gave peaks at *m/e* 446, 428, 411, 410, 399, 303, 272, 271, 254, and 253, which coincided well with those in the spectrum of authentic methyl 3α,7α,12α-trihydroxy-5β-cholestanoate. The mass fragmentation pattern

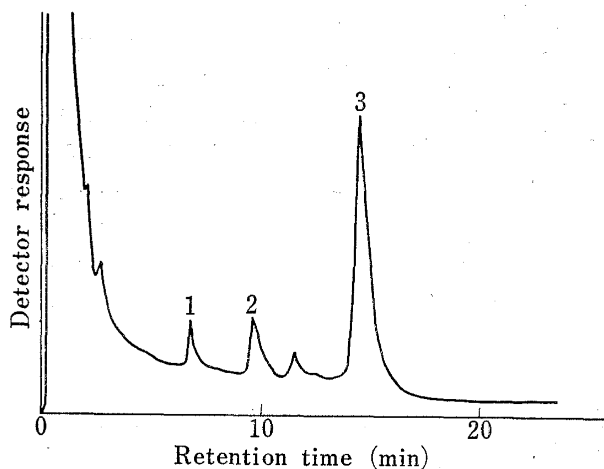

Fig. 2. Gas Chromatogram of the Trifluoroacetyl "Acids" Methyl Esters of *Xenopus laevis*

column: 1% QF-1; column temperature: 240°
The compounds responsible for the peaks 1, 2, and 3 were identified as the derivatives of chenodeoxychoic acid, cholic acid, and 3α,7α,12α-trihydroxy-5β-cholestanoic acid, respectively.

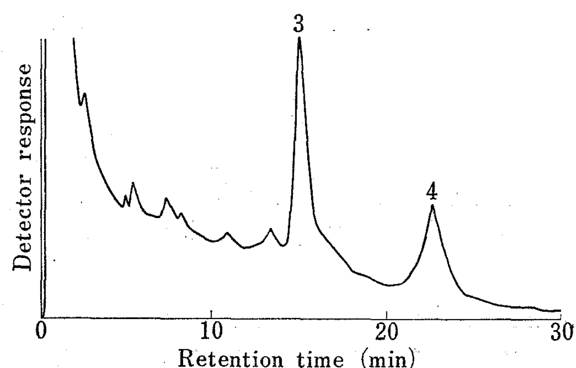

Fig. 3. Gas Chromatogram of the Trifluoroacetyl "Acids" Methyl Esters of *Bombina orientalis*

column: 1% QF-1; column temperature: 240°
The compounds responsible for the peaks 3 and 4 were identified as the derivatives of 3α,7α,12α-trihydroxy- and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acids, respectively.

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(peaks: m/e 386, 368, 357, 271, 261, 254, and 253) of the third component which was recovered from the area corresponding chromatographically to methyl cholate, was identical with that of authentic methyl cholate.

Xenopus laevis has been called a primitive Anura. It might therefore be expected to have bile salts of an evolutionary primitive type. Indeed, the main bile salt of this toad was 5β-cyprinol sulfate, one of the primitive bile salts, but the bile also contained C_{24}-bile acids, cholic acid and chenodeoxycholic acid, though in smaller amounts, so that the enzymes capable of degrading the cholesterol side chain were already present in this primitive Anura.

2. Bile Salts of *Bombina orientalis*

An examination of the bile salts of this Anura by TLC showed spots due to free bile acids but no spot at the place characteristic of conjugated bile acids or bile alcohol sulfates.

The bile salts were dissolved in water, acidified with diluted hydrochloric acid, and extracted with ether. The extract was washed with water until neutral and concentrated to dryness, leaving an acidic residue (11.0 mg from the bile of 10 gall-bladders). A part of the residue was converted into the trifluoroacetate-methyl ester which was analyzed by GLC (Fig. 3).

Peaks 3 and 4 had the identical relative retention times corresponding to the derivatives of $3\alpha,7\alpha,12\alpha$-trihydroxy- and $3\alpha,7\alpha,12\alpha,24$-tetrahydroxy-$5\beta$-cholestanoic acids, respectively. For mass spectral analysis, the residue was methylated and then separated into two components by preparative TLC with the solvent system EA-2. The first component which moved on the plate with the mobility of methyl $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanoate had an mass spectrum identical to that of authentic methyl $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanoate. The second component had the mobility identical to methyl $3\alpha,7\alpha,12\alpha,24$-tetrahydroxy-$5\beta$-cholestanoate on the plate, and its mass spectrum gave peaks at m/e 444, 426, 356, 341, 338, 327, 271, and 253, which coincided with those in the spectrum of the authentic sample of methyl $3\alpha,7\alpha,12\alpha,24$-tetrahydroxy-$5\beta$-cholestanoate.

The nature of the bile salts of *Bombina orientalis* is curious in a physiological sense, because the frog has only unconjugated higher bile acids which can be considered as inefficient detergents than conjugated bile acids or bile alcohol sulfates. Higher bile acids often occur as the unconjugated form in amphibian bile, but these usually contain bile alcohol sulfates as their chief bile constituents. The present finding is the first example of a vertebrate lacking both conjugated bile acids and bile alcohol sulfates.

Another interesting feature is that the higher bile acids of *Bombina orientalis* were identical with intermediates in the cholic acid biogenesis from cholesterol in mammals. It was previously reported that $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanoic acid was formed from cholesterol and efficiently converted into cholic acid in patients with bile fistula.[11] It was also evident that $3\alpha,7\alpha,12\alpha,24$-tetrahydroxy-$5\beta$-cholestanoic acid was made from $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanoic acid by rat liver preparations and was converted into cholic acid in bile fistula guinea pig.[12] Thus, *Bombina orientalis* may be said to have evolved to the stage of $3\alpha,7\alpha,12\alpha,24$-tetrahydroxy-$5\beta$-cholestanoic acid in bile salt evolution but not further than this acid.

3. Bile Salts of *Hyla arborea japonica*

TLC of the bile salts of this Anura showed the presence of a material, as its predominating constituent, with the same mobility as the simultaneously run 5β-ranol sulfate. No spot was observed at the positions characteristic of conjugated and free bile acids. Acid hydrolysis of the bile salts gave "neutrals" (1.4 mg from the bile of 5 gall-bladders).

11) J.B. Carey, Jr., *J. Clin. Invest.*, **43**, 1443 (1964).
12) *a*) T. Masui and E. Staple, *J. Biol. Chem.*, **241**, 3889 (1966); *b*) Y. Tanaka, *Hiroshima J. Med. Sci.*, **14**, 203 (1965).

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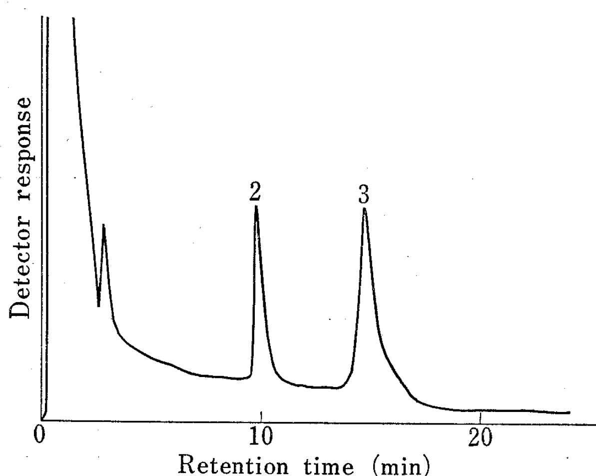

Fig. 4. Gas Chromatogram of the Trifluoroacetyl "Acids" Methyl Esters of *Rana brevipoda*

column, column temperature, and peak identity as in Fig. 2

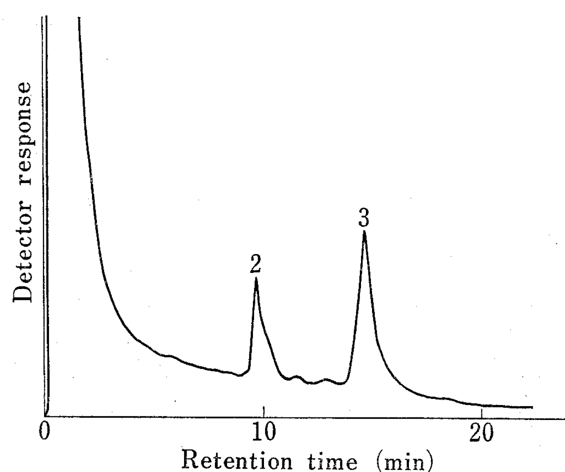

Fig. 5. Gas Chromatogram of the Trifluoroacetyl "Acids" Methyl Esters of *Rana limnocharis limnocharis*

column, column temperature, and peak identity as in Fig. 2

When examined by TLC in the solvent system CE-2 or EAW-2, the "neutrals" gave a single spot which had the *Rf* value of 5β-ranol. After purification by preparative TLC, the main component of the "neutrals" was identified as 5β-ranol by GLC (as the trimethylsilyl ether on OV-1 or OV-17 column) and by mass spectrometry (peaks: m/e 420, 402, 384, 369, 339, 312, 289, 271, and 253).

The bile salt pattern of *Hyla arborea japonica* closely resembled those of some Ranidae; *Rana pipiens* and *Rana catesbiana* contained 5β-ranol sulfate as the principal or a sole bile salt.[3] One may speculated that the present finding is an indication of the relationship between Hylidae and Ranidae.

4. Bile Salts of *Rana brevipoda*

Eight gall-blidders of this Anura gave 13.0 mg of crude bile salts, TLC of which showed the presence of materials with the mobilities corresponding to taurotrihydroxycholanoates, taurotrihydroxycholestanoates, cholestanepentol sulfates, and free bile acids. Hydrolysis of the bile salts gave "neutrals" (5.7 mg) and "acids" (3.2 mg).

From the analysis by TLC and GLC, it was concluded that 5β-cyprinol was almost the sole component of the "neutrals". Identity of the natural bile alcohol with 5β-cyprinol was established by mass spectrometry.

GLC (Fig. 4) showed that the "acids" consisted chiefly of two components with the relative retention times corresponding to cholic acid and 3α,7α,12α,-trihydroxy-5β-cholestanoic acid, and TLC and mass spectrometry confirmed their identity.

The bile salt pattern of this frog closely resembled that of *Rana nigromaculata*, which had also 5β-cyprinol, cholic acid, and 3α,7α,12α-trihydroxy-5β-cholestanoic acid.[13] Such findings agree with generally accepted idea about the two species of Ranidae: that they are closely related.

5. Bile Salts of *Rana rugosa*

The bile salts (129.5 mg) were obtained from 100 gall-bladders of this Anura. When examined by TLC, the bile salts gave a principal spot at the place characteristic of cholestanepentol sulfates and a faint spot at the position of taurotrihydroxycholestanoates. After hydrolysis, "neutrals" weighing 105.9 mg and "acids" weighing 2.5 mg were obtained.

13) T. Kazuno, S. Betsuki, Y. Tanaka, and T. Hoshita, *J. Biochem.* (Tokyo), **58**, 243 (1965).

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From the analysis by TLC (CE-2, EAW-2) and GLC (as the trifluoroacetate or trimethylsilyl ether on QF-1, OV-1, or OV-17 column), it was concluded that the "neutrals" appeared chromatographically to consist almost entirely of 5α-cyprinol. The "neutrals" fraction was crystallized from acetone to give crystals with mp 242°, undepressed on admixture with authentic 5α-cyprinol. The infrared spectrum and mass spectrum (peaks: *m/e* 434, 416, 387, 368, 313, 307, 299, 291, 289, 272, 271, and 253) of the crystals were identical with those of authentic 5α-cyprinol.

The "acids" gave a spot with the polarity of 3α,7α,12α-trihydroxy-5α-cholestanoic acid on TLC with the solvent system S-7, and after conversion into the trifluoroacetate-methyl ester it gave a single peak which had the same relative retention time as the trifluoroacetate of methyl 3α,7α,12α,-trihydroxy-5α-cholestanoate on GLC in QF-1 column.

The nature of the bile salts of *Rana rugosa* is similar except in the configuration at C-5, to that of *Rana nigromaculata*. Thus, enzyme systems responsible for bile salt biosynthesis in the former must be similar to those of the latter. The only difference between them seemed to be the reductases for stereospecific reduction leading to 5α- or 5β-configuration. The same situation has been observed between *Rana temporaria* and *Rana pipiens*.[3] The former contained 5α-ranol sulfate as its chief bile salt, while the latter 5β-ranol sulfate.

6. Bile Salts of Rana *limnocharis limnocharis*

TLC of the bile salts of this Anura showed the presence of materials with the polarities of taurotrihydroxycholanoates, taurotrihydroxycholestanoates, cholestanepentol sulfates, and free bile acids. After hydrolysis, "neutrals" weighing 1.4 mg and "acids" weighing 0.8 mg were obtained from the bile of 4 gall-bladders.

After preparation of the trimethylsilyl ether, the "neutrals" was analyzed by GLC on OV-1 or OV-17 column, and its data showed that 5β-bufol was the only bile alcohol present in this frog. More rigorous evidence for the identity was obtained by mass spectrometry. The natural bile alcohol had an identical mass spectrum (peaks: *m/e* 416, 398, 385, 383, 380, 369, 368, 367, 271, and 253) as that of authentic 5β-bufol.

Fig. 5 shows a typical gas chromatographic recording of the trifluoroacetate-methyl ester of the "acids". Two main peaks were seen. The faster peak had the relative retention time of the trifluoroacetate of methyl cholate, and the slower, the trifluoroacetate of methyl 3α,7α,12α-trihydroxy-5β-cholestanoate. The methylated "acids" were subjected to preparative TLC in the solvent system EA-2, and the esters were recovered from the areas chromatographically corresponding to methyl cholate and methyl 3α,7α,12α-trihydroxy-5β-cholestanoate, respectively. Their mass spectral date confirmed their identity.

Chemical evidence of the bile salts of *Rana limnocharis limnocharis* clearly distinguished this frog from the other Ranidae so far examined by the presence of 5β-bufol sulfate. 5β-Bufol occurs in the toad, *Bufo vulgaris formosus*, as its main bile alcohol,[14] but it has never been found in other species. The present finding of 5β-bufol suggests the interrelationship of the Ranidae and the Bufonidae.

Thus, the Ranidae have different bile alcohols although the bile acids are similar; most of them have cholic acid and 3α,7α,12α-trihydroxy-5β-cholestanoic acid, or their 5α-epimers. They may be divided into three groups on the basis of their biochemical differences in the hydroxylation pattern of the side chain; (a) *R. temporaria*, *R. pipiens*, and *R. catesbiana* have mainly 5α- or 5β-ranol sulfate; (b) *R. nigromaculata*, *R. brevipoda*, and *R. rugosa*, 5α- or 5β-cyprinol; (c) *R. l. limnocharis*, 5β-bufol. It is obvious that these differences can be used taxonomically.

From the facts described in this paper, we can conclude that the bile salts of Anura are primitive. They consist largely of bile alcohols and/or higher bile acids having 26 or 27 carbon

14) K. Okuda, T. Hoshita, and T. Kazuno, *J. Biochem.* (Tokyo), **51**, 48 (1962).

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atoms, which can be considered as biogenetically nearer to cholesterol (C_{27}) than are common (C_{24}) bile acids. Haslewood[3] has suggested that the conversion of cholesterol to C_{24} bile acids in mammals may represent a recapitulation of the evolution of bile salts, and that some of the primitive bile salts could be intermediates in this process. In accordance with this hypothesis, all the higher bile acids of the Anura examined in the present work have been shown to be intermediates in the biosynthesis of the C_{24} bile acids. On the other hand, the main bile alcohols of these Anura cannot be considered as evolutionary or biosynthetic precursors of the C_{24} bile acids, and appear to represent major end products of cholesterol catabolism in these primitive animals. It would be, however, noticed that the structures of the bile alcohols, with exception of ranol, are exactly those that could arise by single hydroxylation of the side chain in 5α- or 5β-cholestane-3α,7α,12α,26-tetrol, which is identified as a precursor of allocholic or cholic acid. It seems easier to believe, therefore, that the bile alcohols arise from the tetrol; it has, in fact, been confirmed in carp[15] that 5α-cyprinol is formed from the 5α-tetrol. In summary, the chemical nature of the Anura bile alcohols suggests that until the stage of the intermediary formation of the tetrol the sequence of reactions in the biosynthesis of bile alcohols are the same as those in the process of cholesterol to C_{24} bile acids found in mammals, and that the tetrol probably represent a branching point of the biosynthetic pathway leading to either bile acids or to bile alcohols.

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15) T. Hoshita, *J. Biochem.* (Tokyo), **66**, 313 (1969).

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