Comparison of the Bile Salts of Frogs with those of their Tadpoles

BILE-SALT CHANGES DURING THE METAMORPHOSIS OF *RANA CATESBEIANA* SHAW

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1. The bile salts of three frog species of the genus *Ptychadena* and of *Rana catesbeiana* have been compared with those of their tadpoles. For *R. catesbeiana* comparison was made of the bile salts in at least ten of the recognized stages of tadpole metamorphosis. 2. In all cases, adult bile salts were more complex than those of the tadpoles. 3. In *R. catesbeiana* after stage 18, 26-deoxy-5 α -ranol was hydroxylated to form 5 α -ranol (27-nor-5 α -cholestane-3 α ,7 α ,12 α ,24 ξ ,26-pentol) and at least two other bile alcohols appeared in solvolysed bile salts. 4. Tadpole bile salts were not found to be biochemically more primitive than those of fully metamorphosed frogs; in some, but not all, cases tadpole bile alcohols could be regarded as biochemical precursors of those in the adult frogs. 5. Detailed evidence for the structure of the bile salts from mass-spectral fragmentation patterns has been deposited as Supplementary Publication SUP 50097 (2 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) 169, 5.

The chief bile salts of amphibians are more varied than those of any other vertebrate group and include C₂₇, C₂₆ and C₂₅ alcohols conjugated with sulphate, unconjugated C₂₈ and C₂₇ acids and C₂₇ and C₂₄ acids conjugated with taurine (e.g. Anderson *et al.*, 1974; Kihara *et al.*, 1977). They thus comprise substances characteristic of primitive as well as of advanced vertebrates and also the sulphates of the ranols and 26-deoxyranols that may be regarded as amphibian specializations (Haslewood, 1978). There is no evidence that any amphibian bile salts are secondary, i.e. not directly made in the liver from sterols. We thought that it would be of interest to compare the bile salts of tadpoles (larval amphibians) with those of the corresponding fully developed frogs to try to find out whether more evolutionarily primitive molecules were present in the earlier stages of development and also what biochemical alterations might occur during tadpole life.

We have compared the bile salts of tadpoles and those of adult frogs for three West African species of the genus *Ptychadena* (Ranidae). With the American bullfrog *Rana catesbeiana* Shaw, we have been able to examine tadpole bile salts in at least ten of the developmental stages delineated by Taylor & Kollros (1946) and to compare the results with those from frogs that had undergone complete metamorphosis. We had previously investigated the *Ptychadena*

adults (Anderson *et al.*, 1974) and have now repeated this examination. Japanese workers have on a number of occasions reported on the bile salts of *R. catesbeiana* (Noma *et al.*, 1976).

Experimental

General

Methods for collection of bile, isolation of bile salts, solvolysis, g.l.c. and t.l.c. were those of Anderson *et al.* (1974) or Haslewood & Haslewood (1976), except where otherwise stated. Because of shortage of material and since it was already known that the bile salts of the frog species examined consist chiefly of alcohol sulphates or (in one case) unconjugated C₂₇ acids, alkaline hydrolysis was not done. Bile acids and alcohols were identified by mass spectrometry and also by comparison of the g.l.c. retention times of their esters and/or trimethylsilyl derivatives with those of known compounds from our collection. Mass spectra from g.l.c. peaks were compared with those of known compounds where these were available; other structures were deduced from fragmentation patterns. These patterns were discussed in general by Anderson *et al.* (1974). Detailed evidence for the structure of the bile salts from mass-spectral fragmentation patterns has been deposited as Sup-

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G.l.c.-mass spectrometry of R. catesbeiana products

The gas-chromatographic separations were performed on a U-shaped column (length 1.8 m, internal diam. 2 mm), packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories Inc., State College, PA, U.S.A.), mounted in a Hewlett-Packard 402 gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.). The column and detector temperatures were kept at 250–260°C and 280°C respectively, and the helium flow rate was 30 ml/min. The remaining details of g.l.c.-computerized mass spectrometry were as described by Anderson *et al.* (1974). The volatile trimethylsilyl derivatives were prepared by leaving the solvolyzed bile-salt mixtures (0.5–1 mg) for 1 h at 60°C with *NO*-bis(trimethylsilyl)trifluoroacetamide (50 µl) containing 1% (w/v) chlorotrimethylsilane and trimethylsilylimidazole (10 µl). Silylating reagents were from Pierce Chemical Co., Rockford, IL, U.S.A. The reaction mixtures were stored at room temperature overnight. The volumes of the solutions were reduced to approximately 30 µl by evaporation under a stream of N₂. Portions of the resulting mixtures were injected into the gas chromatograph. G.l.c. retention times for substances listed in Table 2 increased in the order *K, H, J, N, L, E, O*.

Bile salts from Ptychadena species

The tadpoles were obtained artificially by fertilization *in vitro* in the Department of Zoology, University of Ibadan, Ibadan, Nigeria. Adults of three species, namely *Ptychadena (P.) oxyrhynchus* Smith, *Ptychadena maccarthyensis* (Andersson) and *Ptychadena mascareniensis* Dumeril and Bibron, were collected at night in a temporarily flooded area of 'farm bush' (a cultivated area in the interphase between savanna and forest) at Ibadan in August, 1974. Vocal characteristics supplemented morphological means of identification. Some captured female frogs of each species had begun to ovulate (as determined by palpation). Later the same night eggs expressed from these females were exposed to a suspension of sperms prepared by mascerating testes of conspecific males. The resulting tadpoles were raised in the laboratory in shallow enamel dishes on a diet of boiled lettuce supplemented by Bemax (Vitamins Co., Brentford, Middx., U.K.). Specimens for dissection were taken at a variety of ages and anaesthetized with an aqueous solution (500 mg/litre) of tricaine methanesulphonate (MS222; Sandoz, Basle, Switzerland). Gall bladders were immediately removed and stored in an excess of

ethanol. Adults from the same site were similarly anaesthetized, dissected and their gall bladders were stored in ethanol. The adults sampled included the parents of the above tadpoles. Yields were as follows (number of individuals, mg of bile salts): *P. oxyrhynchus* tadpoles (20, 4.8); *P. oxyrhynchus* adults (9, 4.5); *P. maccarthyensis* tadpoles (15, 5.2); *P. maccarthyensis* adults (3, 5.3); *P. mascareniensis* tadpoles (30, 3.8); *P. mascareniensis* adults (7, 7.4).

Collection of bile from R. catesbeiana

The tadpoles, from a fish hatchery near Lake Hefner, Oklahoma City, OK, U.S.A., were identified as those of *R. catesbeiana* by Professor C. C. Carpenter of the University of Oklahoma. They were kept in the laboratory in an aerated tank and fed on chopped spinach and/or boiled lettuce. After about stages 18–20, they ate little. Metamorphosis ceased during winter months and was resumed in the spring. Before dissection, the tadpoles were anaesthetized for 30–60 min in an aqueous solution (200 mg/litre) of tricaine methanesulphonate (Ayerst Laboratories, New York, NY, U.S.A.). Gall bladders were stored in methanol. For preparation of bile salts, gall bladders were broken up under methanol and the supernatant drawn off with a pipette. The residue was rinsed with a little fresh methanol and the combined methanol extracts cleared by low-speed centrifugation. Precipitated material was rinsed once with methanol and the combined methanol extracts evaporated to dryness, finally *in vacuo*. Dry residues were rinsed with light petroleum (b.p. 30–60°C) with very little loss in weight. Details of bile salts so obtained are shown in Table 1.

Results

The distribution of substances more or less completely identified by g.l.c. and g.l.c.-mass spectrometry is shown in Table 2. In addition, we detected a small amount of 5β-ranol, the 5β-epimer of substance *E* (Table 2), in sample *K* (stage 25+, i.e. adult) from *R. catesbeiana*.

The definition of major or minor constituents is as used by Anderson *et al.* (1974). We also compared solvolyzed bile salts from tadpoles and adults of the frog *Plectimantis leonardi* (Boulenger); the g.l.c. profiles were somewhat different, but mass-spectral identification was not sufficiently good for us to draw useful conclusions. We examined all bile-salt samples by t.l.c. The results showed differences between tadpole and frog bile salts and confirmed that almost the sole bile salt in tadpoles of *P. oxyrhynchus* is trihydroxycoprostanate (acid = *M*, Table 2). T.l.c. also demonstrated the appearance of more polar substances in samples *F–K* from *R. catesbeiana*, corresponding to the progressive increase in these samples of the sulphate of 5α-ranol (*E*, Table 2).

Table 1. Detail of developing *R. catesbeiana* tadpoles, their gall bladders and bile salts

Sample no.	Stages (Taylor & Kollros, 1946)	Number of individuals	Average wt. (g)	Average length (cm)	Average body length (cm)	Average wet wt. of gall bladders (mg)	Final dry wt. of bile salts (mg)
A	3, 4, 5	7	1.2	4.8	1.8	122	8.5
B	6, 7, 8	12	2.3	6.0	2.3	235	20.7
C	12	1	6.7	8.9	3.6	58	3.3
D	15	1	8.1	9.6	3.6	44	2.9
E	18	1	10.0	9.7	3.7	22	0.8
F	19	2	12.2	11.1	3.8	76	4.5
G	20	1	14.3	11.5	4.2	97	9.2
H	21	1	8.0	9.4	3.7	69	7.8
I	22½	1	6.4	8.7	3.8	88	10.4
J	23	1	5.1	8.6	3.9	64	6.8
K	25+	1	9.1	10.8	4.5	114	13.2

Table 2. Bile alcohols and bile acids detected by g.l.c. and g.l.c.-mass spectrometry after solvolysis of frog and tadpole bile salts

Substances are lettered A–O and are: A, 5 α -bufol (5 α -cholestane-3 α ,7 α ,12 α ,25 ξ ,26-pentol); B, 5 β -bufol (the 5 β -epimer of A); C, 5 α -cyprinol (5 α -cholestane-3 α ,7 α ,12 α ,26,27-pentol); D, 5 β -cyprinol (the 5 β -epimer of C); E, 5 α -ranol (27-nor-5 α -cholestane-3 α ,7 α ,12 α ,24 ξ ,26-pentol, formula II); F, 27-deoxy-5 α -cyprinol; G, 27-deoxy-5 β -cyprinol; H, 26-deoxy-5 α -ranol (formula I); I, 26-deoxy-5 β -ranol; J, 27-nor-5 α -cholestane-3 ξ ,7 α ,12 α ,25-tetrol; K, 26,27-bisnor-5 α -cholestane-3 ξ ,7 α ,12 α ,24-tetrol; L (probably) 27-nor-5 α -cholest-23-ene-3 ξ ,7 α ,12 α ,26-tetrol; M, trihydroxycoprostanic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholestan-26- or -27-oic acid); N (probably) a 5 α -cholestane-3,7,12,x-tetrol; O, alcohol(s) of unknown constitution. X indicates a major constituent and x indicates a minor constituent.

Frog species	Presence of bile alcohols or bile acids														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
<i>P. oxyrhynchus</i>															
Tadpoles													X		
Adults		x		X			x		x				x		x
<i>P. maccarthyensis</i>															
Tadpoles				x			X								
Adults		x		x			X		x						x
<i>P. mascareniensis</i>															
Tadpoles								X		x		x			
Adults	x		X		x	X		X							x
<i>R. catesbeiana</i> samples															
[stages (see Table 1)]															
A (3, 4, 5)								X		X	X	x		x	
B (6, 7, 8)								X		X	X	x		x	
C (12)								X		x	X			x	
D (15)								X		x	X				
E (18)								X		x	X				
F (19)					x			X		X	X	x		x	x
G (20)					x			X		X	X	x		x	x
H (21)								X		X	X	x		x	x
I (22½)					X			X		X	X	x		x	X
J (23)					X			X		X	X	x			X
K (25+)					X			X		x	X	x		x	x

Discussion

The most obvious feature of our results is that the bile-salt mixture is more complex in later stages of frog metamorphosis. In every case (Table 2), a greater

variety of molecules was found in the adult frogs than in the tadpoles; this presumably means that more enzymes are expressed as development proceeds. A particularly striking case is that of *P. oxyrhynchus*, in the tadpoles of which we could find only a single

substance acting as a bile salt whereas at least six were present in the adults; the corresponding numbers of bile salts for *P. maccarthyensis* and *P. mascareniensis* were two and five and three and six respectively. The tadpoles of *R. catesbeiana* contained three to five bile alcohols up to stage 18, and seven or more in subsequent stages. It is not always clear how the substances in adult bile could have arisen from those in the tadpoles, for example in *P. oxyrhynchus* 5 β -bufol, 5 β -cyprinol and 27-deoxy-5 β -cyprinol (*B*, *D* and *G* in Table 2) can hardly be made by biosynthesis from trihydroxycoprostanic acid (*M*, Table 2), although 26-deoxy-5 β -ranol (*I*, Table 2) could have arisen from this acid (Betsuki, 1966). In *P. maccarthyensis* 5 β -bufol (*B*) and 5 β -cyprinol (*D*) could have arisen by hydroxylation at C-25 and C-27 respectively from 27-deoxy-5 β -cyprinol (*G*); there is no evidence that 27-deoxy-5 β -cyprinol is a precursor of the ranols. In *P. mascareniensis* 26-deoxy-5 α -ranol (*H*) is an obvious biochemical precursor of 5 α -ranol (*E*), but not of any of the other adult bile alcohols.

In *R. catesbeiana* the situation is much clearer for from stage 19 onwards 5 α -ranol (*E*, formula II) is present as well as its probable precursor 26-deoxy-5 β -ranol (*H*, formula I). Moreover, the proportion of 5 α -ranol increases with development, so that we can say with fair certainty that at stage 19, defined by Taylor & Kollros (1946) as the stage at which the skin window becomes clear before forelimb protrusion in an animal still having a full tail and with well developed hind limbs, an enzyme system hydroxylating 26-deoxy-5 α -ranol at C-26 comes into play and is thereafter increased in effect up to adult life.

The partially identified substance *N* and other alcohols of unknown structure also increase from stage 19 onwards in *R. catesbeiana*, supporting the view that this is a stage at which biochemical changes become particularly evident.

We cannot say that any of the tadpole bile salts are more primitive biochemically than those of the

adults; thus there is no evidence in our cases of 'recapitulation', i.e. of embryology repeating phylogeny at the molecular level.

Deuchar (1966) has reviewed the biochemistry of amphibian development and reports work with *R. catesbeiana* showing developmental changes in haemoglobins, eye pigments and enzymes of the urea cycle. The ability to secrete urea rather than ammonium ion is regarded as an adaptation to terrestrial life. Alterations in bile-salt biosynthesis might be the result of adaptations to changes in diet during and after metamorphosis; all our species are omnivorous as tadpoles, but mainly insectivorous as adults.

One of the striking ecological features of the tropics and sub-tropics is high species diversity. This is associated with high niche diversity and frequently with phenotypic plasticity. It is well exemplified by the genus *Ptychadena*, which contains a variety of similar forms living in apparently similar habitats. *P. oxyrhynchus* and *P. mascareniensis*, for instance, overlap broadly in the forest and forest-savanna interphase. They are similar morphologically, both with particularly streamlined bodies; they breed in similar aquatic sites at similar times of year. In the laboratory as adults and tadpoles they can be maintained on identical diets. *P. maccarthyensis* is morphologically almost indistinguishable from *P. mascareniensis*; they even exhibit similar intra-specific polychromatism. *P. maccarthyensis* is characteristically a savanna species, but overlaps with the other two species where forest meets savanna; indeed the present material was all obtained from the same circumscribed breeding site. Interspecific genetic isolation is ensured by behavioural means; females have the ability to discriminate between the mating calls of different males, but even the vocal patterns show structural similarities. Perhaps the most likely method of niche separation is variation in diet (on which no direct evidence is available) and this is also the aspect of ecology most likely to be correlated with bile-salt chemistry. The current evidence of diverse tadpole bile-salt structure might therefore reflect subtle dietary niche variation amongst these sibling species. Such variation in the larval stage adds an extra dimension to a search for niche separation in adults. Alternatively, the inter-specific bile-salt variation might simply be a further expression of the genetic variability already noticed in the sub-family Raninae (Anderson *et al.*, 1974).

Bile-salt changes during development have been studied in mammals. The human infant has a bile-salt pattern that is different from that of the adult and some of its bile acids may arise by an alternative biochemical route; the same may be true of other animals, for example rabbits (for review see Haslewood, 1978). More primitive bile acids or alcohols have not been detected in embryonic life.

(I) 26-Deoxy-5 α -ranol (R = H) (II) 5 α -Ranol (R = OH)

Finally, it is of interest to compare our present findings with previous work on adult frogs. In addition to the substances now detected, Anderson *et al.* (1974) found minor amounts of 26-deoxy-5 α -ranol in *P. maccarthyensis* and of 5 β -bufol and 26-deoxy-5 β -ranol in *P. mascareniensis*; such small differences are not unusual in single species. Noma *et al.* (1976) solvolysed the bile salts from a collection of 200 specimens of *R. catesbeiana* by the dioxan/trichloroacetic acid method and, after chromatography, isolated seven bile alcohols. These were identified as 5 α -cyprinol, 5 α - and 5 β -ranol and the four 26-deoxy-5 α - and -5 β -ranols epimeric at C-24. We did not detect 26-deoxy-5 β -ranol in our material, but of course we used only a single specimen (*K*, Table 1) of the fully metamorphosed frog. We were surprised, however, to find that 5 α - rather than 5 β -ranol was a principal bile salt in *R. catesbeiana*, for Noma *et al.* (1976) and previous Japanese workers have emphasized the preponderance of the 5 β -isomer; we have no explanation for this difference.

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References

- Anderson, I. G., Haslewood, G. A. D., Oldham, R. S., Amos, B. & Tökés, L. (1974) *Biochem. J.* **141**, 485–494
Betsuki, S. (1966) *J. Biochem. (Tokyo)* **60**, 411–416
Deuchar, E. M. (1966) *Biochemical Aspects of Amphibian Development*, pp. 135–139, Methuen, London
Haslewood, G. A. D. (1978) *The Biological Importance of Bile Salts*, pp. 93–96 and 124, North-Holland, Amsterdam
Haslewood, E. S. & Haslewood, G. A. D. (1976) *Biochem. J.* **155**, 401–404
Kihara, K., Yasuhara, M., Kuramoto, T. & Hoshita, T. (1977) *Tetrahedron Lett.* **8**, 687–690
Noma, Y., Noma, Y., Kihara, K., Yasuhara, M., Kuramoto, T. & Hoshita, T. (1976) *Chem. Pharm. Bull.* **24**, 2686–2691
Taylor, A. C. & Kollros, J. J. (1946) *Anat. Rec.* **94**, 7–23