A More Detailed Study of Bile Salt Evolution, Including Techniques for Small-Scale Identification and their Application to Amphibian Biles

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1. Methods have been developed for the isolation and identification of small amounts of bile salts and of bile acids and alcohols obtained by solvolysis. These methods involve preparative and analytical t.l.c., purification on columns of protonated Al_2O_3 and Sephadex LH-20 and also g.l.c.–mass spectroscopy of solvolysis products. 2. Application to 29 species of frogs and toads has confirmed the constancy of bile salt patterns in a single species, including colour phases in two instances, and has revealed great variations between different species in some genera (e.g. *Rana*, *Ptychadena*) and little difference between widely distributed species in others (e.g. *Bufo*). 3. Taxonomic deductions should be made with caution and with regard to the physiological significance of the biochemical character considered. The molecular differences found might be interpreted as indicating variations in the rate of evolution.

Studies of the bile salts of many vertebrate species have resulted in the almost complete elucidation of all chemical problems encountered and also in a concept of bile salt evolution that is both biochemically and taxonomically acceptable (Haslewood, 1971a). The circumstantial evidence derived from biosynthetic and species distribution studies suggests that evolution of the principal bile salts derived from cholesterol has taken the course shown in Scheme 1. One substance of type *B*

(Scheme 1) has been isolated, from lamprey bile (Haslewood & Tökés, 1969); its biochemical affinities are unknown. It is uncertain whether substances of type *C* give rise to C_{24} acids, although this is possible (Danielsson & Kazuno, 1964).

In many vertebrate groups (most teleosts, most lizards, snakes, birds and mammals) bile salt evolution has proceeded to C_{24} acids, so that variations now to be seen comprise no more than differences in the pattern of hydroxylation of these substances.

Scheme 1. Apparent evolutionary relationships of the bile salts

In other groups, molecules of more primitive types are found, apparently without C_{24} acids, suggesting that the animals may have become evolutionarily 'senescent' as far as their bile salts are concerned. In a few animal groups, numerous small morphological and behavioural differences between closely related species suggest that perhaps evolution is very active; such groups can be found, for example, amongst fishes of the families Cyprinidae and Catostomidae and also amongst anuran amphibians (frogs and toads). It so happens that the bile salts both of cyprinids and catostomids (Anderson & Haslewood, 1970) and of anurans show large chemical variations. A few anuran species have already been examined (Haslewood, 1967; Kuramoto *et al.*, 1973) and we refer to some of these below (see the Discussion section).

We have been fortunate in having the opportunity to study the numerous frog and toad species living in Nigeria, and we set out to identify the bile salts of some of these animals and of other related anurans, with the purpose of assessing their value as taxonomic characters and as possible indicators of evolutionary 'vigour' or 'senescence'.

To carry out this work it was necessary to develop new techniques, suitable for the identification of such amounts of bile salts as were available from the few small gall bladders that we could usually collect. We now describe these techniques, their applications to some amphibian biles, the results obtained and our tentative assessment of their significance.

Materials and Methods

General

Solvents (except analytical-quality acetic acid) for chromatography were redistilled at 4–6 weekly intervals; methanol and ethanol were redistilled over solid KOH. T.l.c. plates of silica gel were kept in a sealed container and heated for 30 min at 100°C before use. For loading t.l.c. plates, adding solvents to powdered KBr for i.r. spectroscopy and for general transference of small amounts of liquid we used a 100 μ l g.l.c. syringe (model 100 A-RN, Scientific Glass Engineering Pty. Ltd., Melbourne, Australia) which fitted into an Agla Micrometer Syringe Outfit (Burroughs Wellcome, London N.W.1, U.K.).

Bile salts

Nigerian amphibians were usually captured at night at their aquatic breeding sites. They were kept for periods varying between 1 h and 2 weeks, without food, before being killed with MS 222 (Tricaine methansulphonate, Sandoz Ltd., Basle, Switzerland). The gall bladders were then immediately dissected free and placed in an excess of ethanol. Usually, gall bladders from several frogs were kept together

as a single sample for analysis. Most of the samples were drawn from single localities, but in a few cases (indicated as Western or Mid-Western Nigeria in Table 1) they were drawn from localities separated by as much as 500 miles. For *Arthroleptis* and perhaps also *Hylarana* there are behavioural indications that the populations sampled, although appearing morphologically homogeneous, may include more than one species. Bile salts were partially purified as described by Haslewood (1967).

I.r. spectroscopy

Material (50–200 μ g) in methanol (10 μ l) was put on to powdered KBr (20–25 mg) and dried at 40°C for at least 15 min. The mixture was repowdered and made into a disc (diam. 1.5 mm) in the usual way. Spectra were taken with a model 137 or 157G spectrophotometer (Perkin-Elmer Co. Ltd., Beaconsfield, Bucks., U.K.).

G.l.c.

This was as described by Anderson & Haslewood (1970). Retention times of trimethylsilyl ethers relative to that of methyl trimethylsilyl cholate (1.00) on the column used were: 26-deoxy-26-nor-5 α -ranol, 0.63; 26-deoxy-26-nor-5 β -ranol, 0.71; 26-deoxy-5 α -ranol, 0.77; 26-deoxy-5 β -ranol, 0.88; 27-deoxy-5 α -cyprinol, 1.12; 27-deoxy-5 β -cyprinol, 1.25; 5 α -ranol, 1.40; 5 α -bufol, 1.52; 5 β -ranol, 1.57; 5 β -bufol, 1.72; 5 α -cyprinol, 1.82; arapaimol-A, 1.93; 5 β -cyprinol, 2.04. The values for 27-deoxy-5 α -cyprinol and 26-deoxy-5 α -ranol were deduced from the 5 α –5 β differences determined experimentally in other cases.

G.l.c.–mass spectrometry

The solvolysis products (0.5–2.0 mg) were silylated with an excess of *N*-trimethylsilylimidazole (Pierce Chemical Co., Rockford, Ill., U.S.A.) at 80°C for 1–2 h. Portions (1–2 μ l) of the silylation mixture were analysed directly on a Hewlett-Packard F and M-402 gas chromatograph (Hewlett-Packard, Palo Alto, Calif., U.S.A.) which was interfaced, via an all-glass double-stage Biemann-Watson-type molecular separator (Varian-MAT G.m.b.H., Bremen, Germany), with a Varian-MAT CH-7 mass spectrometer (Varian-MAT G.m.b.H.). When the presence of a bile acid was detected, the analysis was repeated on a fresh sample which was first methylated with diazomethane and then silylated as above.

The g.l.c. separations were carried out on a column [1 m; 3% OV-17 (Applied Science Laboratories Inc., State College, Penn., U.S.A.) or 1.2 m; 3.8% UCW-98 (Hewlett-Packard)] at 235–250°C and 50 ml/min helium flow rate. The g.l.c.–mass spectrometer interface and molecular separator temperatures were kept at 240–250°C. The mass spectra were

measured at 70eV ionizing voltage and at 180°C ion-source temperature. The data collection, unit resolution, mass identification and spectral plotting were carried out on an IBM-1800 computer (IBM Corp., San Francisco, Calif., U.S.A.) equipped with 24K core memory and an IBM Calcomp plotter.

Preparative and analytical t.l.c. for conjugates

Silica gels (Kieselgel G, from E. Merck A.-G., Darmstadt, Germany; MN-Kieselgel G-HR from Machery, Nagel and Co., Düren, Germany and Silica gel CT from Reeve, Angel Scientific Ltd., London E.C.4, U.K.) were tried for preparative t.l.c. in layers (0.5 mm thick) on glass plates (5 cm × 20 cm). All proved satisfactory for periods up to 4 weeks and then appeared to 'age', subsequently giving poorer separations. Precoated plates (0.25 mm thickness, without indicator; E. Merck A.-G.) did not have this disadvantage and gave less residue after elution with methanol.

Bile salts (up to 1 mg) in methanol (50 µl) were loaded in as narrow and even a band as possible at about 1.5 cm from one end of the plate. After equilibration in the usual way for 15 min, the jar containing the plate was tilted so that solvent reached the silica gel. Chromatography was stopped when the front had travelled about 18 cm.

Solvent mixtures were chloroform-methanol-aqueous NH₃ (sp. gr. 0.88, analytical grade), 15:10:1, by vol., or ethyl acetate-acetic acid-water, 15:12:2, by vol. After being dried at room temperature and then at 100°C, the cooled plates were exposed to iodine vapour, the defined zones were scraped off and material was eluted from the silica gel with methanol. The filtered methanolic extracts were evaporated to dryness in a stream of N₂ and the residues washed with a little cold ether, redried and weighed. They were then dissolved in methanol (50 µl) and analysed by t.l.c. as previously described (Haslewood, 1971b) with the mixture, acetic acid-3-methylbutyl acetate-water (7.5:7.5:3 or 9:7:3, by vol.) Standards were (systematic names of steroids are given in the legend to Table 1) purified sodium 5β-bufol sulphate, bile salts of *Rana esculenta* (mainly 5β-cyprinol sulphate), *Rana pipiens* (mainly 5β-ranol sulphate) and *DiscoGLOSSUS pictus* (mainly taurotrihydroxycoprostanate). Preparative t.l.c. with chloroform-methanol-aq. NH₃, as described, separated the three substances used as standards and also separated 5β-cyprinol sulphate from 27-deoxy-5β-cyprinol sulphate and 5β-ranol sulphate from 26-deoxy-5β-ranol sulphate. It did not separate the 5α from the corresponding 5β sulphates, nor did it separate the cyprinol from the bufol sulphates.

Analytical t.l.c. did not differentiate between taurotrihydroxycoprostanate and cyprinol sulphate; otherwise it had about the same discriminatory power

as preparative t.l.c. Nevertheless, 3-methylbutyl acetate-acetic acid-water mixtures were, in our hands, better able to separate taurine conjugates and bile alcohol sulphates than other solvent systems described in the literature.

Purification of conjugates on columns

(a) *On protonated alumina.* Small columns of protonated alumina (Al₂O₃-HCl, 0.1 g) were used as described by Haslewood (1971c); they were conveniently packed by using capillary pipettes to transfer Al₂O₃-HCl suspended in methanol. Conjugates, eluted with methanol from silica gel after preparative t.l.c., were used as the anions on Al₂O₃-HCl. After elution from Al₂O₃-HCl with ammoniacal methanol (Haslewood, 1971c) the products were obtained as follows: (i) the NH₃-water-methanol eluate was evaporated to dryness on a boiling-water bath in a N₂ stream; (ii) excess of aqueous 0.1 M-Na₂CO₃ was added [for the Al₂O₃-HCl sample described by Haslewood (1971c), this was 2 ml of 0.01 M-Na₂CO₃] followed by excess of ethanol and evaporation to dryness was carried out as before; (iii) to the residue was added excess of 0.1 M-aqueous HCl (0.4 ml for 2 ml of 0.01 M-Na₂CO₃ used above) followed by ethanol (excess) and the mixture was evaporated as before; (iv) the residue of NaCl and sodium salts of conjugates was extracted with ethanol (1-2 ml) and the filtered extract evaporated. Repetition of this process (iv) gave the conjugates almost free from chloride and showing little impurity on i.r. spectroscopy, especially when precoated silica-gel plates had been used for preparative t.l.c. This method, involving contact with high concentrations of protons, might be expected to cause decomposition in some cases and, although we did not notice this in the present work, the results should be checked against the original bile salts.

(b) *On Sephadex LH-20.* This column, described by Haslewood & Haslewood (1972), gave conjugates free from major impurities. However, elution of 'blank' columns with chloroform-methanol (1:1, v/v) gave material (0.01-0.02 mg/ml of effluent) that showed very intense absorption in the region 1500-1800 cm⁻¹; hence i.r. spectra of conjugates purified by this method had to be interpreted with caution. The region 625-1400 cm⁻¹, containing bands frequently diagnostic of steroid nuclear hydroxyl substitution patterns and configuration at C-5 (Haslewood, 1967), were not affected.

Solvolysis of sulphates (cf. Burstain & Liebermann, 1958)

Conjugates (1-4 mg) were dissolved, with gentle warming, in 0.1 M-HCl (1 ml). NaCl (200 mg) was added and dissolved; this usually produced turbidity

Table 1. *Bile salts of some anurans*

The bile salts detected are labelled A–R, which are the following: A, 5 α -bufol sulphate [5 α -cholestan-3 α ,7 α ,12 α ,25 ξ ,26-pentol (probably) 26 sulphate, formula I]; B, 5 β -bufol sulphate (the 5 β epimer of compound A); C, 5 α -cyprinol sulphate (5 α -cholestan-3 α ,7 α ,12 α ,26,27-pentol 26 or 27 sulphate, formula II); D, 5 β -cyprinol sulphate (the 5 β epimer of compound C); E, 5 α -ranol sulphate (5 α -27-norcholestan-3 α ,7 α ,12 α , 24 ξ ,26-pentol 24 sulphate, formula III); F, 5 β -ranol sulphate (the 5 β epimer of compound E); G, 27-deoxy-5 α -cyprinol (= 25-deoxy-5 α -bufol) 26 sulphate; H, the 5 β epimer of compound G; I, 26-deoxy-5 α -ranol sulphate; J, the 5 β epimer of compound I; K, 26-deoxy-26-nor-5 α -ranol, formula IV (sulphate); L, the 5 β epimer of compound K; M, taurotrihydroxycoprostanate [taurine-conjugated 3 α ,7 α ,12 α -trihydroxy-5 β -cholestan-26 (or 27)-oic acid, formula V]; N, unconjugated 3 α ,7 α ,12 α -trihydroxy-5 β -cholestan-26 (or 27)-oic acid; O, trihydroxy-bufostercholeonic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholest-22-ene-24-carboxylic acid); P, unconjugated cholic acid; Q, arapaimol-A (5 β ,25 ξ -cholestane-2 β ,3 α ,7 α ,12 α ,27-pentol, formula VI); R, partially identified or unidentified steroids. The evidence for identification was obtained by methods a–e which are as follows: a, analytical t.l.c. with or without preparative t.l.c.; b, i.r. spectroscopy of conjugate; c, g.l.c. of trimethylsilyl bile alcohols after solvolysis; d, g.l.c.–mass spectroscopy of trimethylsilyl bile alcohols or trimethylsilyl ester ethers of acids or methyl ester trimethylsilyl ethers of acids, after solvolysis; e, t.l.c. and i.r. spectroscopy of bile alcohols or acids after solvolysis. X, Chief bile salt; x, minor constituent; n.t., not specifically tested for unconjugated acids.

Family and species	Number of gall bladders in collection (where known) and origin	Bile salts																	
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Discoglossidae																			
<i>Discoglossus pictus</i>	28, Malta		x d		x d		x d				x d			X ab	x a		x ad		
Pipidae					X ab cd										X ad				
<i>X. mulleri</i>	6, Ibadan Western Nigeria				X ab cd				x d		x d				X ad				
Bufonidae																			
<i>Bufo b. formosus</i>	Japan		X ab cd					x d	x cd							x* d			x d
<i>B. b. vulgaris</i>	1, Oxford, England		X ab cd					x cd	X cd						x a				x d
<i>B. maculatus</i>	12, Ibadan Western Nigeria		X ab cd						X cd						x a				x d
<i>B. marinus</i>	Jamaica		X ab cd						X cd						x a				
<i>B. regularis</i>	Ibadan, Western Nigeria		X ab cd						x cd						x a				
Ranidae, Arthrolepinae																			
<i>Arthroleptis (poecilnotus?)</i>	24 Western and mid-western Nigeria		x d		X cd		X ab cd	x ad	X cd		x cd		x d	X ab					
<i>Cardioglossa leucomystax</i>	7, Ijebu-Ode, Western Nigeria								x d	x cd	X cd				n.t. a			X cd	X† ab cd
<i>C. nigromaculata</i>	2, Ijebu-Ode, Western Nigeria														n.t. a				X† ab
Ranidae, Corniferinae																			
<i>Hylarana albolabris</i>	2, Western Nigeria	X cd		X cd				x d		X ac d									

Table 1—continued

Family and species	Number of gall bladders in collection (where known) and origin	Bile salts)																	
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Ranidae, Raninae, <i>Aubria subsigillata</i>	5, Western Nigeria (forest)						X ab cd				X ab cd		x cd		x a				
<i>Dicroglossus occipitalis</i>	9, Ibadan, Western Nigeria (savanna)		x d		X ab cd			x d	X ab cd	x d	x cd		x d	x ab	x ad				x d
<i>Ptychadena aequiplicata</i> (two collections, examined separately)	4 and 9, Western Nigeria (forest)		x d		x cd		x d	x c	X cd	x cd	X ab cd								
<i>P. longirostris</i> (two collections, examined separately)	4 and 10, Western and mid-western Nigeria (forest)		x d		X ab cd				X cd	x cd	X cd								x d
<i>P. maccarthyensis</i>	3, Northern and Eastern Nigeria (savanna)		x d		X ab cd				X ab cd	x cd	X cd								x d
<i>P. mascareniensis</i>	Ibadan, Western Nigeria (forest)	x d	x d	X ab cd	x cd			x d	x d	X cd	x cd								
<i>P. oxyrhynchus</i>	11, Western and Mid-Western Nigeria (savanna)		x d		X ab cd				X cd		x cd				X ad				
<i>P. taenioscelis</i>	3, Ibadan, Western Nigeria (savanna)	X cd		X cd				x d		X cd	d				n.t. a				x d
<i>Rana esculenta</i>	20, Commercial source (Europe)			x cd	X ab cd														x d
<i>R. galamensis</i>	4, Ibadan, Western Nigeria (savanna)	x d		X cd						x cd									
<i>R. pipiens</i> complex	6, Commercial source (North America)						X ab cd				x d	x d	x d						x d
<i>R. ridibunda</i>	5, Kent, England				X ab cd				x cd					X ab e					
<i>R. temporaria</i>	Commercial source (Europe)					X ab cde				X cd									
Rhacophoridae <i>Hyperolius concolor ibadensis</i> (colour phases J1, J2 and F examined separately)	13, 8 and 7 respectively, Western Nigeria (forest-savanna)		x d		X ab cd				X ab cd										X† d
<i>H. fusciventris burtoni</i> (colour phases J1 and F examined separately)	20 and 8 respectively, Western Nigeria (forest)		x d		X ab cd				X ab cd									x d	x† d

Table 1—continued

Family and species	Number of gall bladders in collection (where known) and origin	Bile salts																	
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
<i>H. guttulatus</i> (a mixture of all colour phases and phase J1 examined separately)	4 and 7 respectively, Ibadan, Western Nigeria (forest)		x d		X ab cd			x d	X ab cd									x d	x† d
<i>H. nitidulus</i>	4, Ilorin, Western Nigeria (savanna)				X ab cd				X ab cd									x d	X† d

* This remarkable acid was found by method d only in the bile of *Bufo b. formosus*, from which it had been previously isolated by Japanese workers and finally identified by Hoshita *et al.* (1967). We now confirm their structural assignment.

† The bile salts of two species of *Cardioglossa* appeared quite similar and are discussed in the Results section.

‡ *Hyperolius* samples proved difficult to investigate by g.l.c. probably because of persistent impurities. A number of constituents remain unidentified. The principal unknown substances in *H. concolor ibadensis* and *H. nitidulus* were apparently identical.

or a precipitate. Ethyl acetate (4 ml) was added and, the stoppered mixture was kept, with occasional mixing, at 37–38°C for 48 h. It was then extracted twice with ethyl acetate. The aqueous portion was tested with

0.5M-BaCl₂ (0.4 ml) for SO₄²⁻. The ethyl acetate was washed with water, aqueous NaHCO₃ solution (excess) and water, dried over Na₂SO₄ and evaporated, leaving neutral material (bile alcohols,

etc.) as residue. Solvolysis appeared to be almost quantitative; the method caused some acetylation of the bile alcohols, which was readily detected by g.l.c.-mass spectroscopy. Unconjugated bile acids were not fully removed by this procedure and appeared in the g.l.c.-mass spectra: of course they were retained by omitting the bicarbonate washing.

Results

These are shown in Table 1. The assessment of which were 'chief' bile salts was based on the original t.l.c. and i.r. examination or on subsequent g.l.c. after solvolysis. In most cases, it was easy to decide which were the principal constituents, but in some of the more complex bile salts this judgement must be regarded as tentative. No free bile alcohols were detected by t.l.c. in any biles, but some of the minor constituents seen on g.l.c. after solvolysis might have existed in the free form in the original bile salts. Unconjugated bile acids were present in a number of cases; the condition of collection and storage were such as to make it certain that these could not have arisen by post-mortem hydrolysis.

A few hitherto undiscovered compounds were detected and are chief bile salts in *Cardioglossa leucomystax*. A principal alcohol (present as monosulphate) in this animal is apparently arapaimol-A (Haslewood & Tökés, 1972). The relative retention time of the maximum of the other chief g.l.c. peak given by the trimethylsilyl-solvolysed bile salts might correspond to that of the 5 α epimer of arapaimol-A, but mass spectroscopy shows that a mixture of substances is responsible for this peak. Arapaimol-A was also found as a minor constituent in three species of *Hyperolius*.

A previously unrecognized substance is 26-deoxy-26-nor-5 β -ranol detected, together with minor amounts of its 5 α epimer (formula IV), in a few biles and sufficiently predominant in *Rana pipiens* to suggest that its isolation in substance might be feasible.

Apart from the above-mentioned compounds, the chief bile salts found were the bufol sulphates (5d, formula I), cyprinol sulphates (5d, formula II) and ranol sulphates (5d, formula III), their C-terminal deoxy derivatives and taurotrihydroxycoprostanate (formula V). Some bile salts, e.g. those of *Rana esculenta*, *R. galamensis* and *Discoglossus pictus* showed on t.l.c. faint spots corresponding in mobility to the taurine conjugate of cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid) or its 5 α epimer. The amount of material concerned was too small for isolation or preparative t.l.c. and (for the reasons cited in the Discussion section) we have not attempted a complete analysis of the minor conjugated bile acids.

Discussion

Methodology

Identification of conjugates depends on ability to separate them; this is limited at present by the discriminatory power of preparative t.l.c. I.r.-spectroscopic characterization of 5 α - or 5 β -3 α ,7 α ,12 α -trihydroxy conjugates was convincing and, in addition, we observed that the strong broad sulphate band in the region 1150–1300cm⁻¹ is almost smooth with an -O·SO₃⁻ group, but with the grouping -CH₂·SO₃⁻ (taurine conjugates) there are well-marked shoulders at about 1175 and 1255cm⁻¹. This feature enabled us to judge with confidence whether conjugates consisted wholly or mainly of sulphate esters or taurine conjugates, even when impurities obscured the amide bands at about (max.) 1545 and 1650cm⁻¹. We also noticed that ranol or 26-deoxyranol sulphates showed a well-marked band at about (max.) 946cm⁻¹ (5 α) or 926cm⁻¹ (5 β). This we attribute to interaction between the -O·SO₃⁻ group at C-24 and the hydroxyl group at C-12. Molecular models of ranol sulphates show that these groups are in close proximity and can easily be made to touch.

The evidence for conjugates that we now present is not as good as that for bile alcohols. Methods for hydrolysing conjugates of C₂₇ acids require prolonged heating in concentrated alkali at quite high temperatures and these conditions certainly cause some decomposition. We have therefore postponed complete investigation of the bile acids in the hope that milder methods of hydrolysing the conjugates will become available.

G.l.c. was checked in two laboratories and we think that the combined g.l.c.-mass-spectral evidence for alcohols is excellent. Unconjugated acids in substantial proportions of the bile salts were easily identified by t.l.c. and g.l.c.-mass spectroscopy. In summary, we believe that only minor amounts of conjugated bile acids have escaped detection in this study.

All bile alcohols and acids listed in Table 1 exhibited characteristic fragmentation patterns in their mass spectra and could be identified therefore even in unresolved g.l.c. peaks. The mass values and relative intensities of the fragment ions resulting from the sequential losses of the C-17 side chain and trimethylsilyl alcohols from the steroid nuclei provided clear distinction between the usual C-5 epimeric 3 α ,7 α ,12 α -trihydroxy(*m/e* 253, 343 and 433 ions) and 2 β ,3 α ,7 α ,12 α -tetrahydroxy(*m/e* 251, 341, 431 and 521 ions) skeletons. These considerations alone could be misleading, however, with compounds having (or forming easily) a double bond in the side chain. In these compounds, depending on the position of the double bond, the loss of the side chain is often associated with the transfer of two

hydrogens to the expelled radical (Wyllie & Djerassi, 1968). The fragmentation patterns could not differentiate between certain C-3 epimeric compounds, e.g. 5 α -cyprinol and its 3 β isomer, latimerol.

The size and oxidation pattern of the side chains were evident from the high mass range of the spectra. The fully silylated compounds exhibited mostly very weak molecular ions with stronger peaks owing to the sequential losses of trimethylsilyl alcohols. Characteristic fragment ions can dominate the spectra in the presence of silyloxy groups along the side chain owing to preferential cleavage of a carbon-carbon bond adjacent to the silyloxy groups. These diagnostic cleavages can lead to charged fragments in both high and low mass range of the spectra.

Biological

The already known bile salts found in this study (taurotrihydroxycoprostanate and the monosulphates of the bufols, ranols and cyprinols and their C-terminal deoxy derivatives) are those previously found in amphibian biles. Kuramoto *et al.* (1973) examined bile salts of *Xenopus laevis*, *Bombina orientalis*, *Hyla arborea japonica* and three species of *Rana*. They carried out a dioxan-trichloroacetic acid solvolysis followed by alkaline hydrolysis and then separated the product into neutral and acidic substances. These were separated by t.l.c. and subsequently analysed by g.l.c. and mass spectrometry. These methods are adequate for the identification of the main bile salts (although we have found that dioxan-trichloroacetic acid can cause some decomposition in certain cases) and included an examination for minor amounts of acids which we, for the reasons given above, did not undertake. However, it is unlikely that Kuramoto *et al.* (1973) would have detected the large variety of alcohols found by our direct g.l.c.-mass spectroscopic analysis of the solvolysis products. Kuramoto *et al.* (1973) found 5 β -cyprinol sulphate and taurotrihydroxycoprostanate, as we did, as the chief bile salts in *Xenopus laevis* and also small amounts of cholic acid and chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid). In other anurans (none of which we have examined) they detected taurotrihydroxycoprostanate, 5 β -bufol, 5 α - and 5 β -cyprinol, 5 β -ranol and small amounts of C₂₄ acids. A remarkable finding was that the bile salts of *Bombina orientalis* consisted only of unconjugated trihydroxycoprostanic acid and its C-24 hydroxylated derivative (varanic acid or an isomer), previously found in nature only in lizards of the family Varanidae and in *Heloderma horridum* (Haslewood, 1967), but a known intermediate in the biosynthesis of cholic acid from cholesterol.

The few urodeles (newts and salamanders) previously examined have biles like those of anurans (except of course those of *Bombina orientalis*) and these show resemblances to bile salts of *Latimeria chalumnae* and the lungfishes, which are believed to be the living forms closest to the fish stocks ancestral to anurans and urodeles. It seems unlikely that elucidation of the chemistry of the unidentified compounds, for example in *Cardioglossa*, will much alter this picture. Nothing so far discovered in amphibian bile supports the idea of a separate descent of urodeles and anurans from ancestral fish.

Our new methodology has enabled us to test afresh the variations shown by bile salts in a single species. The examination of two separate collections of *Ptychadena aequiplicatus* and *P. longirostris*, three colour phases of *Hyperolius concolor* and two such phases of *H. fusciventris* (Table 1) all showed that even in animal groups in which there are considerable variations of bile salts, the pattern in a single species exhibits no more than quite small quantitative differences. The relevant g.l.c. 'profiles' in the cases cited above were almost identical. These observations strengthen still further the original hypothesis of our bile salt studies, that the primary bile salt chemistry is species-specific and genetically determined. There are no grounds for suspecting the presence of secondary bile salts (i.e. those modified by intestinal micro-organisms) in amphibian bile.

We were surprised to find that one species, *Ptychadena aequiplicata*, had as its chief bile salts the monosulphates of C₂₇- and C₂₆-tetrols (27-deoxy-5 β -cyprinol and 26-deoxy-5 β -ranol). We had previously thought, and observed, that for C₂₆ and C₂₇ compounds at least five hydroxyl groups were required for a physiologically adequate bile salt.

On morphological grounds the *Pipidae* and *Discoglossidae* are thought to be the two most primitive of the families represented in our sample and also to be closely related. There is a basically primitive skeletal structure but extant species are variously specialized, often in a manner which can be correlated with their current niche. *Discoglossus* and *Xenopus* both have a predominantly aquatic mode of life and show structural physiological and behavioural specializations associated with this. In view of the 'advanced' chemical structure of the bile salts of these two families (Scheme 1) it would appear that the bile salts represent a specialized aspect of their biologies. We are unable, however, to correlate this with their feeding habits. Although two other species which have predominantly aquatic habitat, namely *Dicroglossus occipitalis* and *Rana ridibunda*, share with *Xenopus* the bile salt taurotrihydroxycoprostanate, so also does *Arthroleptis*, the most terrestrially adapted species in our sample. In terms of diet the *Pipidae* are probably the most distinctive members of our sample because, unlike those of the other species,

the tadpoles are planktonic feeders, whereas the adults, also unlike the other species, feed entirely in water. However, apart from taurotrihydroxycoprostanate, the chief bile salt is 5β -cyprinol sulphate and this is widespread throughout the series. Even the combination of the two compounds is not unique (Table 1).

The genus *Bufo* comprises a widespread group of anurans whose biology and evolution has been the subject of a recent comprehensive review (Blair, 1972). Although widespread the genus is relatively homogeneous, its members showing clear resemblances in morphology, ethology and ecology. Several of the species are genetically compatible. The bile salts reflect this homogeneity (Table 1).

In the Ranidae our three samples from the subfamily Arthroleptinae are all small forest species with specialized life cycles. They lay heavily yolked eggs and in one of them, *Arthroleptis*, development is direct, there being no free-living tadpole stage. In all three the bile salts are also specialized, either in their structure or diversity.

Our single example of the subfamily Corniferinae is similar to most ranids in morphology and breeding biology but differs in its arboreal habitat and associated digital modifications. The bile salts are also similar to those of some of the Raninae.

Amongst our examples of the Raninae, *Aubria* and *Dicroglossus* are both large predominantly aquatic frogs, the former a forest and the latter a savanna species. Both have distinctive bile salt combinations.

The genus *Ptychadena* differs from *Rana* mainly in the structure of the pectoral girdle. In other aspects of their biology the two genera are basically very similar. The members of both, however, display a series of specific variations, particularly in terms of habitat adaptations and behavioural isolating mechanisms, which separate them ecologically and genetically and which suggest that there has been recent evolution. A good example of this is the North American '*Rana pipiens*' complex, which has recently been shown to consist of at least four biological species, almost indistinguishable morphologically (Littlejohn & Oldham, 1968). Intraspecific variation is also striking in populations of some of the species; colour polymorphism in *Ptychadena mascareniensis* may be cited as an example. The range of bile salt variation in the two genera is quite striking. Of the 11 examples in our sample almost every one is distinctive, although there are clear biochemical relationships within the genus *Ptychadena*. As noted above, this variation does not extend to the population level, for separate samples of *P. aequiplicata* and *P. longirostris* showed identical bile salts.

Our small sample of four African species from the Rhacophoridae all belong to a genus which Schiøtz

(1967), on grounds similar to those cited above, suggests is in a stage of rapid current evolution. Marked colour polymorphism exists in all four species. They are adapted to arboreal life and have habitats ranging from forest to savanna. The four species show a broadly similar bile salt pattern but all have unidentified components and three contain arapaimol-A. Clearly data are needed for more members of the family but on the basis of our small sample it appears that the genus may show characteristics between those of the stable *Bufo* and variable *Ptychadena* and *Rana*.

It is difficult to compare anura at the family level because it is not understood to what extent the Ranidae, for instance, are polyphyletic. At the level of genus there is disagreement as to the groupings and some authors (for example Inger, 1968) group *Dicroglossus*, *Hyla* and *Ptychadena* as *Rana*. The system used in Table 1 has the advantage for our present purposes of grouping together frogs which show clear morphological resemblances. The most striking feature of our findings is the range of interspecific variability in the different genera. The bile salts in some genera (e.g. *Bufo*) seem to vary little and in others (*Rana*, *Ptychadena*) show large interspecific differences.

These results may indicate that the existing phylogenetic classification, particularly in the family Ranidae, is erroneous, or the observed differences may be a reflexion of differences in the rates of bile salt evolution; in some genera such as *Bufo* this may have slowed or ceased, whereas in others such as *Rana* it may still be very active.

Unfortunately the aspect of anuran physiology most relevant to bile salt chemistry, namely the diet, is little investigated, although in a general sense it probably varies in relation to the habitat. It is also possible that the larval diet is the important variable, but on this there is no information. Although, in general, 'modernization' of the bile salts goes hand in hand with advances in the level of organization of the phenotype, the rate of this process must be affected by the problems of fat digestion and absorption that have to be solved and cannot be expected to be the same in every animal group. If our results do indeed indicate that bile salt evolution is more active in some genera than in others, any taxonomic interpretation of this kind of biochemical evidence must obviously be made with caution. For example, the bile salts of *Discoglossus pictus* (mainly taurotrihydroxycoprostanate) are biochemically more advanced than those of *Rana esculenta* (chiefly 5β -cyprinol sulphate) (Scheme 1). However, it would not be justifiable to conclude from this fact that the whole phenotype of the former frog is more advanced than that of the latter. Each biochemical character to be used in taxonomy must be considered on its merits, having regard to the selective forces

affecting its rate of evolution. It is our opinion that the evidence afforded by studies of bile salts, and the suggestions about rates of evolution, will be valuable for evolutionary and taxonomic studies when considered in conjunction with other taxonomic parameters. Wallace *et al.* (1971, 1973) examined the serum albumins, fibrinopeptides, haemoglobins and DNA of frogs of the genus *Rana* and found unexpectedly large differences between species. Their general finding of great molecular diversity is fully supported by bile salt studies. If, however, the bile salt chemistry does indeed indicate rapid evolution in some genera but not in others, it is difficult to understand how any time-scale can be applied to molecular differences, as suggested by Wallace and his colleagues.

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