Bile Salts of the Lungfishes *Lepidosiren*, *Neoceratodus* and *Protopterus* and those of the Coelacanth *Latimeria chalumnae* Smith

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1. Bile salts of the coelacanth *Latimeria chalumnae* Smith (five specimens) and of the three living genera of lungfish (Dipnoi) were examined as completely as possible and compared.
2. The small 'bile acid' fractions include no more than traces of well-known C₂₇ or C₂₄ acids (free or conjugated) and the functioning bile salts must be regarded as alcohol sulphates.
3. Comparison of the alcohols suggest that (a) *Latimeria* stands biochemically outside the animal group which includes the Dipnoi, (b) *Protopterus* and *Lepidosiren* are more closely related to one another than either is to *Neoceratodus*, (c) all four primitive osteichtheans have some amphibian affinities, (d) there are affinities between *Latimeria* and Dipnoi and ostariophysan families (especially Cyprinidae and Catostomidae) and (e) there are biochemical links between Dipnoi and lampreys.

The known most primitive living Osteichtheans (bony fishes) are the coelacanth *Latimeria chalumnae* Smith, discovered in 1938, and the lungfishes (Dipnoi). The latter are represented by three genera, *Lepidosiren* (one species, *Lepidosiren paradoxa* Natterer) from South America, *Neoceratodus* (one species, *Neoceratodus forsteri* Krefft) from Australia, and *Protopterus* (four quite closely related species) from Africa.

These animals comprise the living examples morphologically nearest to the extinct fish stock from which amphibians arose; they are also of considerable interest in themselves as surviving ancient forms. We therefore decided to enquire whether the nature of their bile salts could throw light on their relationships to one another and to other groups of fishes and extant amphibians. The bile salts of *Latimeria* have already been shown to include latimerol sulphate [5α -cholestane- $3\beta,7\alpha,12\alpha,26,27$ -pentol (I) 26 (or 27)-sulphate] with minor amounts of its 3α -epimer, 5α -cyprinol sulphate (Anderson & Haslewood, 1964); we have now examined *Latimeria* more closely, together with *Lepidosiren*, *Neoceratodus* and *Protopterus aethiopicus* Heckel.

In what follows, the term 'bile salts' refers to the ions presumed to be physiologically active in lipid digestion and absorption. In fishes and amphibians, these may be sulphate esters of bile alcohols, taurine conjugates of bile acids or the ions of bile acids themselves. All these substances will be obtained as salts, mainly with Na⁺ or K⁺, after isolation from bile. Solvolysis of sulphate esters liberates bile alcohols and alkaline hydrolysis of conjugates gives bile acids. Changes *post-mortem* or disease in living animals

might lead to enzymic hydrolysis of bile acid conjugates, in which case the bile would contain bile acid ions. However, unconjugated bile acids have been found in apparently healthy amphibians and when changes *post-mortem* have been excluded by the conditions of collection (Anderson *et al.*, 1974).

Methods

Preparation of bile salts, analytical t.l.c., preliminary g.l.c., g.l.c.–mass spectroscopy and solvolysis were as described by Anderson *et al.* (1974) except where otherwise stated. In addition to the g.l.c.–mass spectroscopy conditions of Anderson *et al.* (1974), the complex 'acid fractions' isolated after alkaline hydrolysis were analysed also in chemical ionization mode by using helium or methane as both carrier and reagent gas. These analyses were performed on a Finnigan-3200 computerized g.l.c.–mass spectrometer (Finnigan Corp., Sunnyvale, CA, U.S.A.). Alkaline hydrolysis was done at bile-salt concentrations of 30–50 mg/ml in 0.25 M-NaOH in a

sealed metal bomb at 130–135°C for 16–17 h. Higher concentrations of NaOH resulted in gel formation. After hydrolysis, the contents of the bomb were diluted and the mixture was filtered and acidified with 2M-HCl. The insoluble products were collected by filtration or by extraction with ethyl acetate. The collected precipitate or extract was washed with water. Washed ethyl acetate extracts were dried over Na₂SO₄ and evaporated; washed precipitates were dissolved in methanol and the filtered solutions evaporated. Approximate percentages (w/w of the bile salts) of 'acid' fractions so prepared were: *Latimeria*, 2.0; *Lepidosiren* 7.0; *Neoceratodus*, 2.4; *Protopterus*, 10.0.

Results

The bile alcohols detected by g.l.c. and g.l.c.-mass spectroscopy after solvolysis of the bile salts are listed in Table 1.

T.l.c. of the bile salts by the method of Haslewood & Haslewood (1976) and with the solvent system 2,2,4-trimethylpentane/ethyl acetate/acetic acid (7:12:3, by vol.) showed in *Neoceratodus* no spots corresponding to unconjugated bile acids, in *Latimeria* and *Lepidosiren* traces only, and in *Protopterus* faint spots. The latter had *R_F* values (a) intermediate between those of cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid) and 3 α ,7 α ,12 α -trihydroxy-5 β -cholestan-26-oic acid, (b) slightly less than that of cholic acid and (c) intermediate between this and zero. These results could mean that some or all of the very small amounts of bile acids present are unconjugated. Indeed we obtained no convincing evidence for the presence of bile acid conjugates. The 'acid fractions' prepared after alkaline hydrolysis showed on t.l.c. no spots corresponding to cholic acid

or chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid) or their 5 α -epimers, and no more than a trace, in *Protopterus* only, of substances with the *R_F* of 3 α ,7 α ,12 α -trihydroxy-5 β -cholestan-26-oic acid or its 5 α -epimer. By g.l.c. and g.l.c.-mass spectroscopy these 'acid fractions' proved difficult to analyse and evidently contained neutral material, perhaps carried as micelles with the largely unhydrolysed bile alcohol sulphates. Further details of hydrolysed material which we believe to be acidic are as follows. *Latimeria* yielded g.l.c.-mass spectroscopy peaks suggesting the presence of small amounts of trihydroxy- and dihydroxy-cholan-24-oic acids. *Lepidosiren* contains what appears to be a dihydroxy-cholan-24-oic acid and a dihydroxy-24-norcholan-23-oic acid. *Neoceratodus* yielded traces of what might be a trihydroxycholan-24-oic acid and also a compound whose fragmentation spectrum could be interpreted as arising from a 3 α ,7 α ,12 α ,24-tetrahydroxycholestan-27-oic acid. The mass spectra of *Protopterus* 'acid fraction' suggested the presence of a side-chain unsaturated trihydroxycholeenoic acid, a trihydroxycholestenic acid and the methyl and ethyl homologues of trihydroxycholestenic acids as well as a saturated ethyl homologue of a trihydroxycholestanic acid. Such unsaturation might have arisen by dehydration of tetrahydroxy acids during the prolonged alkaline hydrolysis.

Discussion

It is difficult to assess the biological significance of the acids tentatively identified in the small fractions isolated after alkaline hydrolysis. In a predator such as *Latimeria*, cholic acid and chenodeoxycholic acid, which are common constituents of the bile of teleostean prey, might be taken in the diet and excreted

Table 1. *Bile alcohols from a coelacanth and lungfishes*

Substances detected in solvolysed bile salts are labelled A–I and are the following: A, 5 α -bufol (5 α -cholestane-3 α ,7 α ,12 α ,25 ζ ,26-pentol); B, 5 α -chimaerol (II, 5 α -cholestane-3 α ,7 α ,12 α ,24 ζ ,26-pentol); C, 5 α -cyprinol (5 α -cholestane-3 α ,7 α ,12 α ,26,27-pentol); D, latimerol (I, 5 α -cholestane-3 β ,7 α ,12 α ,26,27-pentol); E, 5 α -petromyzonol (III, 5 α -cholane-3 α ,7 α ,12 α ,24-tetrol); F, 5 β -petromyzonol (the 5 β -epimer of compound E; the g.l.c.-mass spectrometry results were identical with those from an authentic sample prepared by LiAlH₄ reduction of methyl cholate); G, 26-deoxy-5 α -chimaerol [a novel bile alcohol, which gave diagnostic mass-spectral peaks (spectra available from either Laboratory on request)]; H, 27-deoxy-5 α -cyprinol (IV); I, unidentified substances. X, Chief constituent (as sulphate) of the bile salts; x, minor constituent (as sulphate) of the bile salts.

Species	A	B	C	D	E	F	G	H	I
<i>Latimeria chalumnae</i>	x		x*	X					x
(five separate specimens)									
<i>Lepidosiren paradoxa</i>	X		X					X	x
<i>Neoceratodus forsteri</i>	x	X			x		x	X	x
<i>Protopterus aethiopicus</i>			X		x	x		x	x

* Identified by retention time only in this study.

into the gall bladder. Alternatively the presence of C_{27} and C_{24} bile acids in small amounts might indicate the evolutionary beginnings of the biosynthetic pathways usual in more advanced animal forms.

The monosulphates of the bile alcohols listed in Table 1 must be regarded as the functioning bile salts: their possible biological significance may be discussed in question-answer form.

(1) *How similar are the bile salts of lungfishes and of Latimeria to one another?*

Latimeria is quite different from any lungfish, indeed from any other animal so far investigated, in having the sulphate of latimerol (I). Although latimerol is by far the principal bile alcohol, the presence of a small amount of its 3 α -epimer (5 α -cyprinol) may probably be taken as evidence that evolution to 5 α -cyprinol is, or has been, taking place, on the grounds that 5 α -cyprinol is a bile alcohol of more advanced animal forms and that the 3 α configuration is universal in bile salts except for those of *Latimeria* and of the extremely primitive hagfishes (Myxinoidea).

Among the lungfishes themselves, *Lepidosiren* and *Protopterus* have 5 α -cyprinol as a chief bile alcohol but not the 5 α -chimaerol (II) or its C-26 deoxy derivative characteristic of *Neoceratodus*. However, *Lepidosiren* and *Neoceratodus* both have 5 α -bufol, and *Neoceratodus* and *Protopterus* both have 5 α -petromyzonol (III).

All three lungfishes have 27-deoxy-5 α -cyprinol (IV), which may be regarded as a biochemical precursor of 5 α -bufol, 5 α -cyprinol and 5 α -chimaerol.

In summary, *Neoceratodus* has the ability, absent from the other two lungfishes, to make a C-24-hydroxylated C_{27} bile alcohol. This, in our opinion, divides *Neoceratodus* biochemically from *Lepidosiren* and *Protopterus*. The presence of 5 α -bufol and 5 α -petromyzonol, on the other hand, does link *Neoceratodus* with both *Lepidosiren* and *Protopterus*. The two latter forms are probably more closely related to one another, as indicated by their bile salts, than *Neoceratodus* is to either of them.

(2) *To what extent does the nature of the bile salts support the view that amphibians were derived from fish stocks quite closely related to Latimeria or the lungfishes?*

Latimeria bile does not resemble that of any amphibians so far examined, as latimerol has not been detected in these forms. Of the lungfishes, *Lepidosiren* has two chief bile salts, the sulphates of 5 α -bufol and 5 α -cyprinol, that are principal bile salts in a newt *Diemictylus pyrrhogaster* and a salamander *Megalobatrachus japonicus* respectively (see Tammar, 1974b); *Protopterus* likewise has 5 α -cyprinol sulphate as a principal bile salt. On the other hand *Neoceratodus*, with 5 α -chimaerol sulphate as its chief bile salt, is unlike any amphibian investigated. The ranol sulphates, C_{26} substances commonly found in amphibian (especially anuran) bile, were not detected in lungfishes and may represent a specialization of the amphibia; probably several enzymic steps are necessary to make them from C_{27} precursors (Betsuki, 1966). Many anurans so far examined have 5 β rather than 5 α steroids as bile salts (Anderson *et al.*, 1974) and this, too, emphasizes their differences from lungfishes.

In summary, *Lepidosiren* and *Protopterus* bile does resemble that of two caudatan amphibians and also some anurans, and all four primitive osteichtheans contain alcohols found in amphibian bile.

(3) *To what extent do Latimeria and lungfish bile salts resemble those of other bony fishes (Osteichthyes)?*

Members of the ostariophysan families Cyprinidae and Catostomidae strongly resemble the lungfishes in their bile salts. All the cyprinids investigated so far (about 12 species) have 5 α -cyprinol sulphate as the principal bile salt (see Tammar, 1974a) and at least some catostomids have 5 α -chimaerol sulphate in this role (Anderson & Haslewood, 1970; Bussjaeger, 1973). However, both cyprinid and catostomid bile salts also contain some taurine-conjugated C₂₄ acids (e.g. Briggs & Bussjaeger, 1972) and this is a biochemical advance hardly detectable in lungfishes. The biochemical ability to convert latimerol into 5 α -cyprinol (inversion at C-3) is already present, as remarked above, in *Latimeria*. It is tempting to point out to systematists the obvious biochemical links between *Latimeria* and the lungfish on the one hand and some ostariophysan families on the other. No other group of teleosts so far examined shows these similarities, except that lesser amounts of 5 α -cyprinol have been found in some other ostariophysan families (e.g. Cobitidae, Siluridae).

(4) *Are there any other obvious biochemical affinities between the bile salts now examined and those of other animals forms?*

There is a link between lungfish bile salts and those of lampreys. The latter animals, specialized survivors of the agnathan fishes, have 5 α -petromyzonol sulphate as perhaps their sole bile salt (Haslewood & Tökes, 1969). This substance (III) now appears as a minor constituent in *Neoceratodus* and *Protopterus* (which also has the 5 β -epimer, not previously found in

Nature) and must surely be taken as further evidence of the primitive biochemical nature of these fishes and of their links with ancient forms.

The answers to the above questions in our opinion demonstrate once again the almost uncanny correspondence between the nature of bile salts and the systematic positions of the animal forms. It is hard indeed to believe that this correspondence does not have significance for students of evolution.

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