review

Bile salt evolution

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ABSTRACT Viewed against the background of known or supposed biosynthetic pathways for cholic and chenodeoxycholic acids in man and laboratory animals, the chemical nature of bile salts in more primitive animals clearly indicates that evolution from C_{27}, 5α-alcohol sulfates to C_{24}, 5β-acids has taken place. Stages in this evolution, some of which are intermediates in the biosynthesis of C_{24} bile acids, are described for representatives of all the chief vertebrate groups. "Unique" primary C_{24} bile acids may be considered as hydroxylated chenodeoxycholic acids; the possible taxonomic significance of these is discussed. A closer study of the biochemical mechanisms underlying bile salt differences may be expected to throw new light on the nature of the evolutionary process itself.

KEY WORDS bile · bile salts · bile alcohols · bile acids · vertebrate bile · evolution · biosynthesis of bile acids · glycine conjugates · sulfate esters

THE PRESENT STATE of our understanding of cholic acid biosynthesis in mammals can be represented by the sequence *1*→*11* (Fig. 1), with the qualification that the stages between 7α-hydroxycholesterol (*2*) and 3α,7α,12α-trihydroxy-5β-cholestane (*6*) are not well understood (1–3). The chief doubts about these stages concern the point at which the 12α-OH group is introduced ["the substrate for the 12α-hydroxylase" (4)] and at which reduction of the keto group at C-3 occurs to give the 3α-hydroxyl group of cholic acid. Danielsson and Tchen, in their review (1), suggest that an alternative pathway to *4* might lie through 7α,12α-dihydroxycholesterol (*12*), i.e., that another substrate for the 12α-hydroxylase could be 7α-hydroxycholesterol itself. Mendelsohn, Mendelsohn, and Staple (5) consider that 3α,7α-dihydroxy-5β-cholestane (*13*) is an intermediate in bile acid biosynthesis and that 7α,12α-dihydroxy-5β-cholestan-3-one (*5*) is not; if cholic acid is to be made, then *13* may be a substrate for the 12α-hydroxylase.

The sequence given in Fig. 1 is based on the interpretation of the results of experiments in which labeled, presumptive precursors have been administered to animals having biliary fistulae and also of in vitro studies with liver preparations and radioactive compounds that seem related to a supposed biosynthetic pathway. Until recently, no in vitro conversion of cholesterol to bile acids had been reported. However, Mendelsohn, Mendelsohn, and Staple (6) and Mitropoulos and Myant (7) have demonstrated that suitable preparations from rat liver can convert cholesterol to bile acids. The first-named authors (6) obtained a maximum conversion of cholesterol to cholic acid of 0.75%, whereas Mitropoulos and Myant have in general obtained bile acids not hydroxylated at C-12. Lithocholic (*14*), chenodeoxycholic (*15*), 3α,6β-dihydroxy-5β-cholanoic, α- and β-muricholic (*16*,*16a*), 3β-hydroxychol-5-enoic (*17*), and 3β-hydroxycholest-5-en-26-oic (*18*) acids were all identified by Mitropoulos and Myant (8) as arising when rat liver mitochondria were incubated with cholesterol-4-^{14}C. These authors also found radioactive lithocholic, chenodeoxycholic, 3α,6β-dihydroxy-5β-cholanoic, and β-muricholic acids in the bile of a rat with a biliary fistula that had been injected intravenously with tritiated 3β-hydroxychol-5-enoic acid. In vitro, this acid (*17*) gave probably lithocholic, chenodeoxycholic, and 3α,6β-dihydroxy-5β-cholanoic acids when incubated with rat liver mitochondria (8).

These remarkable results suggest that a bile acid nucleus can be formed even after shortening of the side chain has occurred. They also confirm earlier conclusions that, in rats at least, 12α-hydroxylation is not possible except in a suitable C_{27} compound and cannot occur

In this article "5β-cholanoic acid" as a systematic name replaces Heinrich Wieland's "cholanic acid" for the stem-acid $C_{24}H_{40}O_2$, it being understood that the —COOH group is at the end of the side-chain.

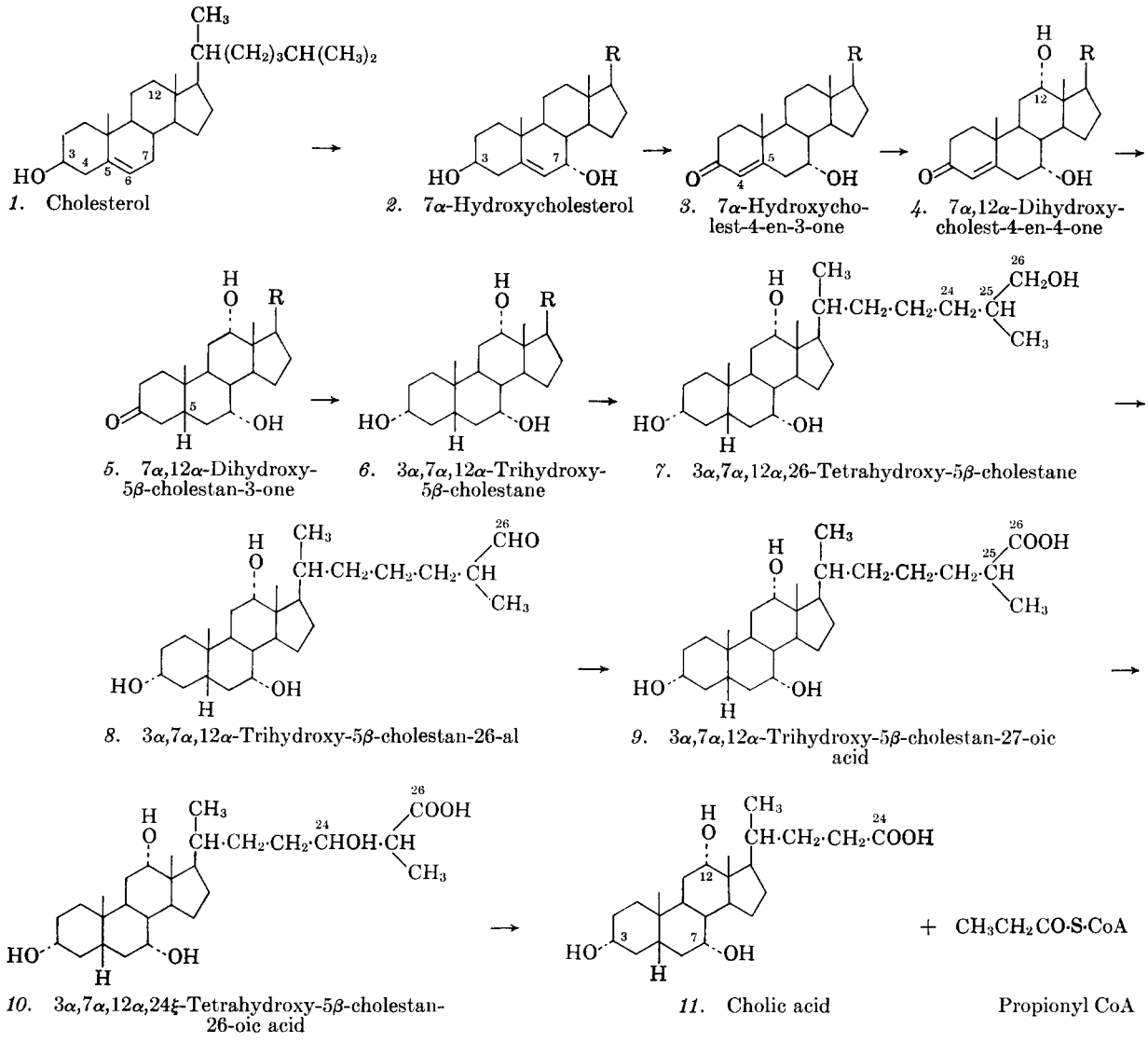


FIG. 1. Proposed stages in the mammalian biosynthesis of cholic acid. R, cholesterol side-chain.

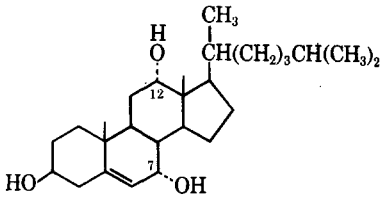
after side-chain shortening. In pythons, chenodeoxycholic acid, *15*, can apparently be a precursor of cholic acid (9).

Danielsson and Tchen (1) express a firmly held view that the biosynthesis of chenodeoxycholic acid might be quite different from that of cholic acid and perhaps the work with in vitro systems will further substantiate this idea.

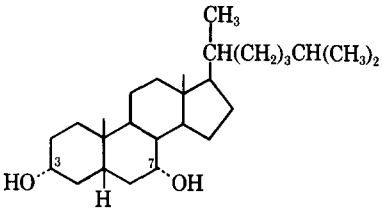
The reason for opening this review with a brief account of the status of our understanding of mammalian bile acid biosynthesis is that this process is a result of evolution and it is now very clear that stages in the sequence given in Fig. 1 can be recognised in animals less highly evolved than mammals. Some of the animals having these more primitive bile salts will now be discussed.

INVERTEBRATES

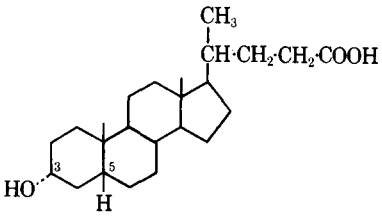
There is no evidence that steroid bile salts occur in invertebrates. The crayfish *Procambarus clarkii* did not have recognisable bile acid conjugates in its digestive juices (10) and Van den Oord, Danielsson, and Ryhage (11) showed that the surface tension-lowering compounds in the gastric secretion of the edible crab *Cancer pagurus* were compounds of fatty acids, sarcosine, and taurine. An example would be decanoyl sarcosyl taurate: $CH_3(CH_2)_8 . CO . N(CH_3) . CH_2 . CO . NH(CH_2)_2 . SO_3^-$. Invertebrates have little or no ability for the biosynthesis



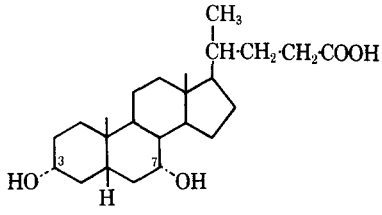
12. 7α,12α-Dihydroxycholesterol



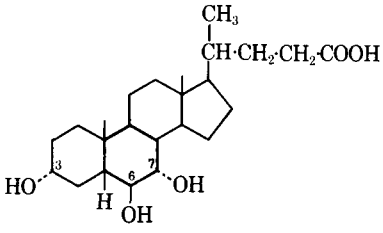
13. 3α,7α-Dihydroxy-5β-cholestane



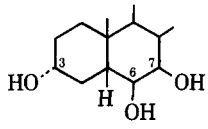
14. Lithocholic acid



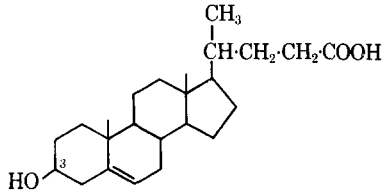
15. Chenodeoxycholic acid



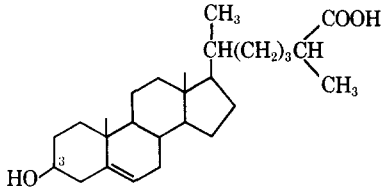
16. α-Muricholic acid



16a. β-Muricholic acid



17. 3β-Hydroxychol-5-enoic acid



18. 3β-Hydroxycholest-5-en-26-oic acid

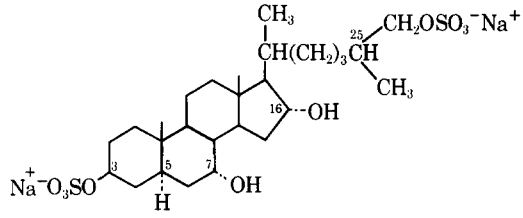
of cholesterol (12) and do not elaborate a bile frankly differentiated as a digestive secretion.

VERTEBRATES

Agnatha

These comprise hagfish and lampreys, both specialized descendants of primitive jawless forms. The two groups are not closely related and the hagfish are considered to be more primitive organisms than lampreys. For example, hagfish (which are entirely marine) have an unmyelinated nervous system, innervate hearts, little or no capacity to make antibodies, and an internal salt composition close to that of sea water (13). Lampreys, by contrast, can adapt to marine or freshwater conditions and do not have the primitive features enumerated for hagfish. Lampreys are parasites as adults, feeding mainly on blood of other fish and, perhaps as a result, the digestive system is degenerate. So far, only enough bile to provide for preliminary examination has been obtained from the small larval forms (ammocoetes). In *Petromyzon marinus* this has shown, chromatographically, a single spot with a 'polarity' slightly less than that of taurocholate and, spectroscopically, that the bile salts are of the allo (5α) type and probably have the 3α,7α,12α-trihydroxy pattern of substitution (14).

Hagfish bile, on the other hand, is plentiful; bile salts, in crystalline form, are easily obtained from it. Two substances at least are present in the crystalline mixture, the most plentiful from two hagfish species being disodium myxinol disulfate which probably has the structure 3β,7α,16α,26(or 27)-tetrahydroxy-5α-cholestane 3,26(or 27)-disulfate (*19*).[1] It was at first supposed (15, 16) that myxinol had the 5β-configuration but further evidence puts the 5α-structure beyond reasonable doubt (17).



19. Disodium myxinol disulfate

[1] The term "26 (or 27)" implies that the configuration at C-25 is unknown. In cholesterol itself, C-26 is regarded as the carbon atom corresponding to the β-methyl group in the mevalonic acid used for biosynthesis. It has been shown (18, 19) that the rat and mouse liver mitochondrial enzymes that oxidize the terminal carbon atoms of the cholesterol side-chain are stereospecific in their attack on C-26 and this probably means that *7* in Fig. 1 is correctly named as 26-ol and *8*, *9*, and *10* as suggested in that figure. The point is undetermined for biosynthesis in other animals, however, and configuration at C-25 is unknown for all bile alcohols.

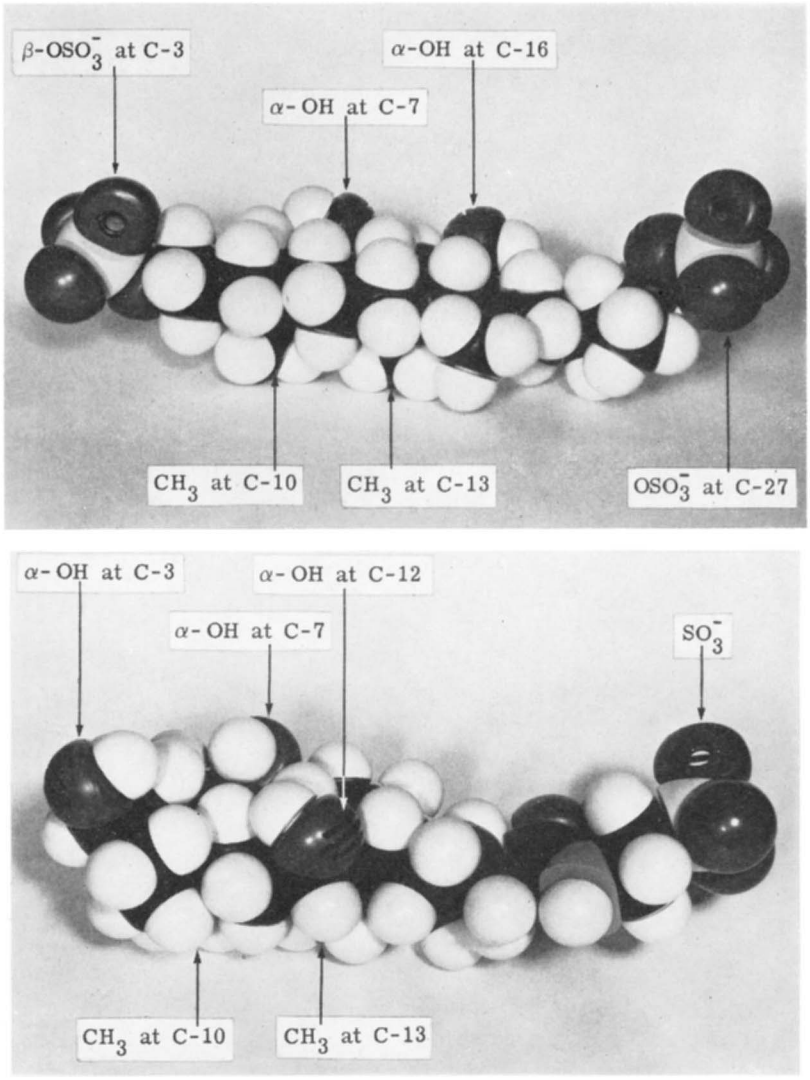


FIG. 2. The most primitive (above) and the most advanced (below) types of bile salt. The photograph above shows a molecular model of myxinol disulfate (formula *19*). The polar SO_3^- and OH groups are not well separated from the lipophilic part of the molecule and cannot be readily made to lie in a plane. The photograph below shows a molecular model of taurocholate [R.CO.NH.CH_2.CH_2.SO_3^-, where R.COOH = cholic acid (formula *11*, Fig. 1)]. The polar SO_3^- and OH groups are sharply separated from the lipophilic part of the molecule and could easily lie in a single plane. The almost equidistant OH groups are so placed that hydration appears likely.

This structure is interesting in several respects. Firstly, it is primitive in that the entire C_{27} skeleton [perhaps derived from cholesterol, the sterol found in *Myxine glutinosa* liver (15)] is present and in that the —OH group at C-3 still has the β-configuration of cholesterol itself (*1*, Fig. 1). Secondly, myxinol shows the 3,7 pattern of hydroxylation, but not the 3,7,12 arrangement of all other bile alcohols. This suggests that stage *1*→*2* (Fig. 1) in bile salt biosynthesis appeared very early in vertebrate evolution, the cholic acid nuclear pattern arising perhaps after other biochemical experiments (of which the 3,7,16 type was one result) had been tried. Thirdly, myxinol is a tetrol; it is the only alcohol with four hydroxyl groups so far discovered acting as part of a principal bile salt. Perhaps this is why two sulfate groups are necessary, a situation also unique in known bile salts. Finally, a molecular model of myxinol disulfate (Fig. 2, top) does not give the impression that the molecule would function very effectively as a detergent; the polar —OH and —SO_3^- groups are not clearly separated from the lipophilic skeleton, as they are, for example, in taurocholate (Fig. 2, bottom). It is hard to escape the conclusion that efficiency as a detergent is of major importance in bile salts. The myxinids apparently function with bile salts that, like other features of their organization, seem to fall far short of

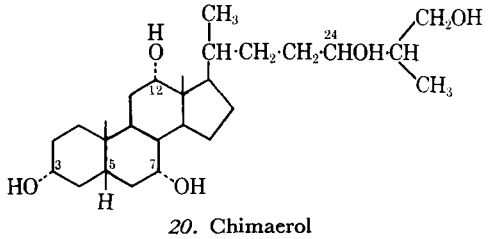
what evolution can accomplish. This point is particularly striking in the hagfishes for these animals are carnivorous, being largely scavengers of dead and dying vertebrates; in more advanced forms there are clear indications that carnivorous habits may have led to "modernization" of bile salts.

The occurrence of the 5α-configuration in the principal bile salts of the two groups of most primitive vertebrates suggests that in bile salt evolution this may indeed be the primitive condition. This 5α-structure is not uncommon in the bile salts of vertebrates (gnathostomes), which are more advanced than the agnathians. Its discovery (first in a C_{24} acid discussed later in this review) was a surprise to workers on bile salts, for the 5α-configuration gives a rather flat nucleus (Fig. 2, top) which was not considered likely to provide as good detergent properties as the L-shaped nucleus that arises from the 5β-structure (Fig. 2, bottom), well-known in the common bile acids.

Chondrichthyes

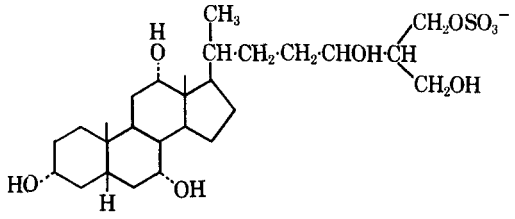
Two main living groups of these fishes can be recognised, namely the Holocephali (rabbitfishes, chimaeras), comprising a few species only, and the highly successful, numerous and diverse sharks and rays (Elasmobranchii; Selachii). The relationships of the chimaeras have recently been discussed paleontologically by Patterson (20), who concludes that "there is evidence of genetic affinity between the holocephalans and the selachians, but no evidence of any direct relationship between the groups."

The bile salt evidence simply suggests that the enzymic systems responsible for bile salt biosynthesis must be closely similar in chimaeras and selachians. The chief bile salt in *Chimaera monstrosa* is the monosulfate of an alcohol that has been called chimaerol (21) and is probably 3α,7α,12α,24ξ,26(or 27)-pentahydroxy-5β-cholestane (*20*).



20. Chimaerol

Chimaerol was detected in a stingray (22) and more recently in the dogfish *Mustelus manazo* (23). Oxidation of the terminal methyl group in *20* would give scymnol (3α,7α,12α,24ξ,26,27-hexahydroxy-5β-cholestane) which, as its 26- or 27-sulfate (*21*), has been found in every selachian examined (24), including *Mustelus manazo* (23). There is probably also some scymnol in *Chimaera monstrosa* bile.



21. Scymnol sulfate

The structures of chimaerol and scymnol differ from those of any other known C_{27} bile alcohol, particularly in the presence of oxygen on C-24. It may be noted, further, that both alcohols have the 5β-configuration, which occurs here apparently to the exclusion of 5α-compounds in a group with a long marine history.

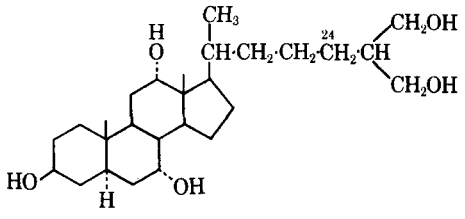
Thus, the nature of their bile alcohols emphasises the isolation from other vertebrate groups and also the close interrelationship of the chimaeras and selachians.

Chimaerol and scymnol could arise by oxidation, at C-24 or C-24 and C-27, respectively, of 3α,7α,12α,26-tetrahydroxy-5β-cholestane (*7*, Fig. 1) and if biosynthesis in these lower groups takes, *mutatis mutandis*, the same course as in mammals, *7* is an acceptable intermediate.

Scymnol, labeled with tritium, proved a poor precursor of cholic acid in rats with biliary fistulae (25) which suggests that it cannot itself be a biosynthetic precursor of cholic acid, although chimaerol, of course, may be. Cholic acid has been found more than once in selachian bile (e.g. 22–24, 26) but a formal demonstration that it can be made by these animals from cholesterol has not been reported. In the writer's laboratory it was detected in the dogfish *Squalus acanthias*, an animal unlikely to have eaten substantial amounts of fishes containing cholic acid. Elasmobranch bile may also contain C_{27} acids.

Osteichthyes

The most primitive living member of the bony fishes is the coelacanth *Latimeria chalumnae* and it fortunately proved possible to elucidate the chemistry of its bile salts. These contain little or no bile acids and the chief alcohol, present as its sulfate, was named latimerol and shown (27) to be 3β,7α,12α,26,27-pentahydroxy-5α-cholestane (*22*).

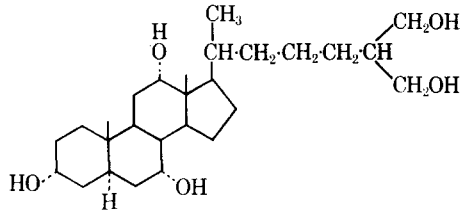


22. Latimerol

The most obvious difference between latimerol and the chondrichthean bile alcohols is the configuration at

C-5. Apart from *Latimeria*, all the osteichthean fishes examined that have bile alcohols of this type are forms with a long freshwater history, and the coelacanths themselves, though now marine, are found as fossils in freshwater deposits.

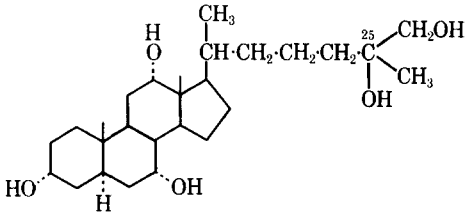
Latimerol, like myxinol, has the C-3β hydroxyl configuration of cholesterol itself. However, *Latimeria* does have enzymes that can invert this group, for a second bile alcohol in this fish is 5α-cyprinol (3α,7α,12α,-26,27-pentahydroxy-5α-cholestane, *23*, a substance present as its C-26 or C-27 sulfate in some other osteichtheans (24) and originally named after its discovery (28) in carp (*Cyprinus carpio*) bile.



23. 5α-Cyprinol

Another primitive Order of bony fishes is the Dipnoi (lungfish) of which there are three genera, namely *Lepidosiren* (South American), *Neoceratodus* (Australian), and *Protopterus* (African).

The bile salts of these fishes have not been fully investigated, but preliminary results show that while *Protopterus* contains a high proportion of 5α-cyprinol, there is also present in *Neoceratodus* the compound 3α, 7α, 12α, 25ξ, 26(or 27) - pentahydroxy - 5α - cholestane (*24*), a substance already named 5α-bufol.



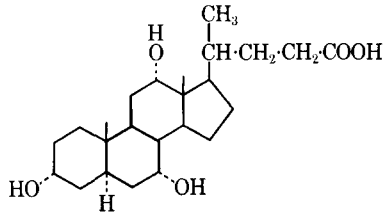
24. 5α-Bufol

These findings arouse speculation as to whether bile salt chemistry can tell us anything about amphibian origins; this possibility is discussed later.

Latimerol was not detected in Dipnoi, examples of all three genera of which have received a preliminary examination.

A puzzling group of Osteichthyes is he Chondrostei, comprising the sturgeons and the American paddlefish (*Polyodon*). These animals, whose fossil history is obscure, are evolutionarily advanced as a whole but show distinct primitive characters.

The bile salts of three sturgeon species, genus *Acipenser*, and of the paddlefish *Polyodon spathula* have been examined (24). The chief bile salt in each case was taurocholate, as in most advanced vertebrates, but allocholic acid (*25*) was also found.

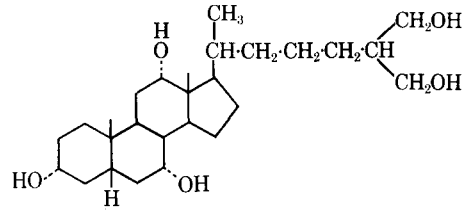


25. Allocholic acid

Allocholic acid (3α,7α,12α-trihydroxy-5α-cholanoic acid) was first obtained by Ohta (29) as an inseparable mixture with cholic acid from the bile of Japanese teleostean fishes; Ohta called his isolated substance "tetrahydroxynorsterocholanic acid" and gave it the formula $C_{27}H_{46}O_6$, later expanded to 3,6,12,x-tetrahydroxy-5β-cholestan-27-oic acid. Later work (30) showed that Ohta's acid was a 2:1 (w/w) mixture of cholic acid (*11*) and its 5α-epimer (*25*). This was the first demonstration of allo compounds in bile salts, referred to above.

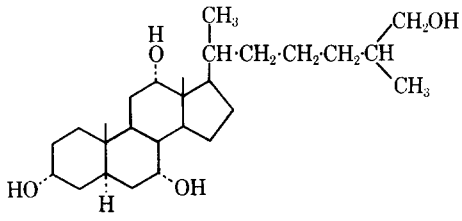
As well as cholic and allocholic acids, chondrostean bile has a small proportion of bile alcohols, consisting of some substances with the 5α- and others with the 5β-configuration. The general properties of the bile alcohol mixture suggest that, as a monosulfate, it is insufficiently hydroxylated to act as an efficient detergent. If such a bile salt mixture were the principal constituent of chondrostean bile, it would not be expected to function effectively. The inference could be drawn that the relict bile alcohols in these animals, having been superseded by more advanced bile acids, have become biochemically nonfunctional and "degenerate," in the same sense as, for example, the useless limb remnants in primitive snakes and other animals that do not make functional use of these organs.

An alcohol identified in the sturgeon *Acipenser huso* is 5β-cyprinol (3α,7α,12α,26,27-pentahydroxy-5β-cholestane, *26*, which constitutes a considerable proportion of the bile alcohol mixture. It is not possible to say whether this means that the sulfate of *26* was once a major bile salt of chondrosteans. 5β-Cyprinol has been found (in traces only) in teleostean fish.



26. 5β-Cyprinol

The principal group of living bony fishes, the teleosts, probably originated from the Holostei, an existing member of which, namely the bowfin *Amia calva*, has been found to have little or nothing except taurocholate in its bile salts. On the other hand a large Order of freshwater teleosts, the Ostariophysi, includes families (Cyprinidae, Catostomidae) whose bile salts consist largely of 5α-cyprinol sulfate with traces of taurocholate (24). Biochemically, the conclusion seems inescapable that cyprinid bile salts are more primitive than those of *Amia* and, indeed, than those of any other teleostean group. In a biosynthetic process that differed from that outlined in Fig. 1 only in the stereospecificity of reduction at C-5 (stages *4* → *5* in Fig. 1), the 5α-alcohol corresponding to *7*, namely 3α,7α,12α,26-tetrahydroxy-5α-cholestane (*27*), would be a precursor of 5α-cyprinol, and indeed *27*, or its C-25 epimer, has been isolated from carp bile (31).



27. 3α,7α,12α,26-Tetrahydroxy-5α-cholestane

Degradation of *27*, as in Fig. 1, would lead to allocholic acid, *25;* to biosynthesize cholic acid it would be necessary to elaborate enzymes having the opposite stereospecificity in the reduction *4* → *5*. This process seems to be complete in *Amia* and in most teleosts, but has barely begun in the cyprinids.

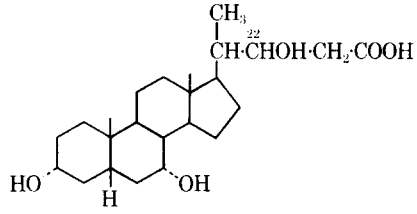
The loach *Misgurnus anguillicaudatus* (family Cobitidae) appears to have 5α-cyprinol sulfate as a principal bile salt with taurocholate as a minor constituent but the silurids examined have principally taurocholate with, in one species at least (*Parasilurus asotus*), 5α-cyprinol in minor amounts (26). *Serrasalmus ternetzi* (the piranha) also has cholic acid (24). It may be that evolution of bile salts in ostariophysans has been stimulated by dietary habits; silurids and the piranha are more carnivorous than the cyprinids so far investigated.

We therefore have the curious situation in which the bile salt chemistry suggests a more primitive organizational level than does the general morphology and paleontology of cyprinid fishes. A recent classification (32) does not put the ostariophysans as the most primitive of the teleosts, so that there is here a clear case for a careful assessment of the value of bile salt chemistry as a taxonomic character.

Numerous non-ostariophysan teleosts have principally taurocholate and taurochenodeoxycholate (the taurine conjugate of *15*) in their bile salts with, in some cases, traces of allocholic acid or of 5β-bile alcohols or C-27 acids. In this respect the eels (Apodes) are especially interesting. The species *Anguilla japonica*, *Conger myriaster*, and *Muraenesox cinereus* have 5β-cyprinol (*26*) and *Anguilla japonica* has two C_{27} acids with the cholic acid substituted ring system (24). Thus these eels have relict biochemically primitive bile salt features; all are carnivorous.

The salmonid *Plecoglossus altivelis* has been reexamined (33) and found to contain a (probably) C_{27} bile acid as a minor constituent; cholic acid is the chief component. Another salmonid (*Oncorhynchus rhodurus*) probably has 3-oxo-7α,12α-dihydroxy-5β-cholanoic as well as cholic acid (26). Herring (*Clupea harengus*) bile has no obvious primitive characters (24).

An interesting finding (34) is that at least some teleosts of the family Haemulidae [Order Perciformes of Greenwood, Rosen, Weitzman, and Myers (32)] have a hitherto undiscovered C_{24} acid, haemulcholic acid (3α,7α,22β-trihydroxy-5β-cholanoic acid, *28*).



28. Haemulcholic acid

Speculations about "unique" C_{24} bile acids are given later in this article.

A grey mullet, *Mugil* species, was found to have almost exclusively taurochenodeoxycholate as a bile salt (24). In mammals this finding would suggest a vegetarian type of diet and indeed the grey mullets are regarded as being specialized for feeding habits of this kind.

In summary, we may say that teleostean fish bile salts are generally advanced but that certain forms have biochemically primitive bile salt types, the significance of which for systematic studies awaits assessment.

AMPHIBIANS

It seems probable that amphibians originated from a group of osteichthean fishes of which *Latimeria* is also a descendant, although some earlier paleontologists had pointed to the Diponi (lungfish) as concerned with amphibian origins. The bile salt evidence certainly relates some living amphibians both to *Latimeria* and to the dipnoans, especially *Neoceratodus*. Thus, the newt *Diemictylus pyrrohogaster* contains bile alcohols (35), one of which is 5α-bufol (*24*), now isolated from *Neoceratodus*. The genus *Diemictylus*, family Salamandridae, is a large and widely-distributed one. Another member of the same family, the European fire salamander

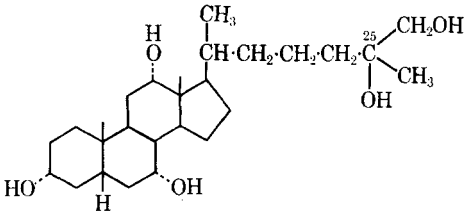
Salamandra salamandra, has a considerable proportion of tauroallocholate in its bile salts; bile alcohols are also present (24).

By contrast, the giant Japanese salamander *Megalobatrachus japonicus* contains 5α-cyprinol as well as a C_{27} trihydroxy bile acid and allocholic acid (36); the last acid was formed after intraperitoneal injection of tritium-labeled 5α-cyprinol (*23*) or 27-deoxy-5α-cyprinol (*27*) into this amphibian (36 *a*).

Thus, the pattern of hydroxylation of the tetrol (*27*) at C-25 or C-27 is common to *Latimeria*, the lungfishes, and at least some newts and salamanders, as well as, of course, to some teleostean fishes.

As mentioned later, allocholic acid is apparently characteristic of many lizards; thus bile salt evolution has taken the same course in some salamanders as in these reptiles, or they are related in this way by descent.

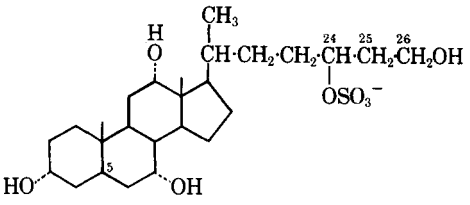
The toads (Bufonidae) also share the C-25-hydroxylating system, for 5β-bufol (*29*) is characteristic of these animals (24).



29. 5β-Bufol

Toads have not been shown to be able to biosynthesize cholic acid, but *Bufo b. formosus* (*Bufo vulgaris japonicus*) could make radioactive 5β-bufol as well as the tetrols (*7*, Fig. 1, and *27*) and the C_{27} acid (*9*, Fig. 1) from cholesterol-4-^{14}C (37). Thus bile salt biosynthesis in toads can also be reconciled with Fig. 1, with additional C-25-hydroxylating enzymic systems and an absence of the final stages. A curious feature of the bile of *Bufo b. formosus* is the presence in it of at least one unsaturated C_{28} acid, trihydroxybufosterocholenic acid, which has the cholic acid ring nucleus. In biosynthetic experiments with cholesterol-4-^{14}C, this substance did not become labeled (37); its origin in toad bile is therefore obscure, though it was claimed in 1937 that toad skin and liver contained C_{28} sterols (38). A recent gas–liquid chromatographic study (38 *a*) of the liver sterols of *Bufo b. formosus* revealed that these consisted of cholesterol and other C_{27} sterols (93%), campesterol (3%), and β-sitosterol (4%).

The bile of frogs of the family Ranidae provided a considerable biochemical surprise, for in two species, *Rana catesbeiana* and *R. temporaria*, the principal bile salts were found (39–41) to be the C-5 epimers of the C-24 sulfate esters of the ranols 3α,7α,12α,24ξ,26-pentahydroxy-27-nor-5α- and -5β-cholestane 24-sulfate, *30*.



30. 5α- and 5β-Ranol sulfate

In intact *R. catesbeiana*, 5β-ranol was found in the bile after intraperitoneal injection of cholesterol-4-^{14}C; there was also isolated radioactive 3α,7α,12α-trihydroxycoprostanic acid,[2] *9*, and a little cholic acid (48). Thus, *R. catesbeiana* bile salts are largely primitive, but biochemical "modernization" can be detected in this animal in contrast to the toad *Bufo b. formosus*, which apparently cannot make cholic acid (37).

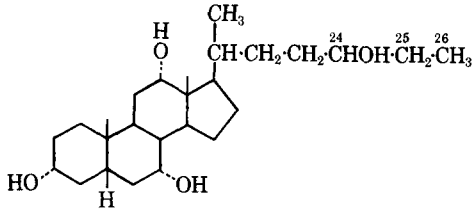
Rana temporaria has mainly the 5α-epimer, 5α-ranol sulfate, in its bile salts (39), but chromatography shows the presence of a substance with the mobility of a 3α,7α,12α-trihydroxycoprostanic acid or its 5α-epimer.

The biosynthesis of the C_{26} ranols clearly indicates a departure from the scheme outlined in Fig. 1, for at some stage a single carbon atom must be lost from the cholesterol molecule. More than one suggestion about a plausible ranol precursor from the substances *2* to *10* can of course be made. Betsuki (49) injected tritium-labeled 3α,7α,12α-trihydroxy-5β-cholestane (*6*) intraperitoneally into specimens of *R. catesbeiana* and isolated from the bile radioactive 5β-ranol, 3α,7α,12α-trihydroxycoprostanic acid (*9*), cholic acid, and also 26-deoxy-5β-ranol (3α,7α,12α,24ξ-tetrahydroxy-27-nor-5β-cholestane, *31*). He suggested that 5β-ranol might arise by decarboxylation of the acid *10* to *31* followed by ω-oxidation of *31*.

5β-Ranol is an efficient precursor of cholic acid in rats with biliary fistulae, unlike 5β-bufol (*29*) and compound *31* (50). 5β-Cyprinol and 3α,7α,12α,24ξ,26-pentahydroxy-5β-cholestane [chimaerol C-24/25 epimer(s)],

[2] The name "coprostanic acid" was given to the stem acid, $C_{27}H_{46}O_2$, of the acid *9*, by agreement between the author and the late Professor T. Shimizu, after the elucidation of its chemistry in 1952 (42). Both epimers at C-25 of *9* have been isolated from *R. catesbeiana* bile and their stereochemistry has been elucidated (42). Both are effective precursors of cholic acid in rats with bile fistulae (43). Their relationship to the systematically named acid *9* is unclear, the difficulty being that in animals (frogs, alligators) that have biosynthesized 3α,7α,12α-trihydroxycoprostanic acid from radioactive cholesterol, the acid was obtained as its conjugate with taurine and this was hydrolyzed by prolonged heating with alkali, which might cause epimerization at C-25. If epimerization did not occur, the biosynthetically made acid is identical (e.g. 44, 45) with the 25α-compound made by one-carbon degradation after anodic coupling of cholic acid, R.COOH, with L(+) 3-methyl glutaric acid, HOOC.CH_2.(CH_3).CH_2.COOH, and so this should correspond to *9*. An efficient mild method for splitting bile acid conjugates is badly needed. Enzymes from *Clostridium perfringens* will hydrolyze taurine and glycine conjugates (46); the glycocholate hydrolase has been purified (47).

tritium-labeled, were also efficient cholic acid precursors in these conditions (50 *a*).



31. 26-Deoxy-5β-ranol

It is likely that a chief bile salt in the American *Rana pipiens* is also 5β-ranol sulfate, but in *Rana nigromaculata* it has been found (51), in contrast to an earlier report, that bile alcohols are present; the chief of these is 5β-cyprinol (*26*). 3α,7α,12α-Trihydroxycoprostanic acid was also found, with a small proportion of cholic acid. When tritium-labeled 5β-cyprinol was injected intraperitoneally into guinea pigs with biliary fistulae, 7% of the radioactivity appeared in the bile; 80% of this was in cholic acid (52). The chief guinea pig bile acid, chenodeoxycholic acid, also became labeled; it is difficult to account for this. The mechanism by which 5β-cyprinol became cholic acid in this experiment might possibly be relevant to the situation in *R. nigromaculata*, but trihydroxycoprostanic acid could hardly be an intermediate.

Thus, the Ranidae are heterogeneous in their bile alcohol chemistry and presumably also in the enzymic processes and hence in the DNA underlying it. The biochemical differences seem great and should afford opportunities for studies that may be of interest in an evolutionary context. The presence of cholic acid perhaps suggests that evolution in this group is active.

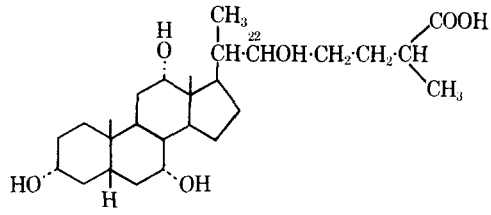
There is little resemblance between the bile salts of Bufonidae and Ranidae: it remains to be seen whether the C-25-hydroxylation pattern common to toads and certain salamanders indicates a common ancestry for bufonids and these tailed forms.

Clearly, much more chemical and biochemical work is needed on amphibian bile.

REPTILES

Bile alcohols have not been found above the organizational level of amphibians.

The bile salt evidence is clearly in accordance with the suggestion (53) that the chelonians (turtles and tortoises) have arisen from a reptilian stock (Diadectomorpha) distinguishable from the rest (Captorhinomorpha) from the earliest fossil reptiles. So far, nothing in common between chelonian bile salts and those of any other vertebrate group has been discovered. In the few cases examined in detail (24), several (probably) taurine-conjugated bile acids have been detected chromatographically, but the only substance whose chemistry is fairly well known is the so-called "tetrahydroxysterocholanic acid" which has been isolated from three chelonian species and is probably (24, 54) one of the C-22 isomers of 3α,7α,12α,22ξ-tetrahydroxy-5β-cholestan-26(or 27)-oic acid, *32*.



32. 3α,7α,12α,22ξ-Tetrahydroxy-5β-cholestan-26(or 27)-oic acid

Biosynthetically, this substance could arise by C-22 hydroxylation of acid *9* and thus by a diversion of the scheme in Fig. 1.

The acid *32*, characterised by its ready formation of a lactone, has not been discovered elsewhere in nature. Japanese workers have claimed (54) that it and other chelonian bile acids occur free (unconjugated) in the bile; in my laboratory we have not detected free bile acids on paper or thin-layer chromatograms in any bile unquestionably collected in a fresh state, except in toads (*Bufo b. bufo*).

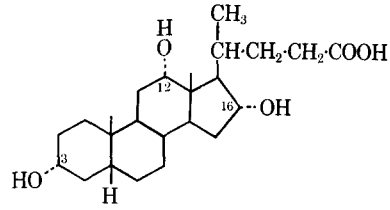
Lizards of the family Iguanidae have largely allo bile acids and the Jamaican anole *Anolis lineatopus* has little but tauroallocholate (the taurine conjugate of *25*) as a bile salt (24). Bile salts of some lizards of the families Iguanidae, Agamidae, and Chameleontidae give on chromatograms spots corresponding in mobility to 3α,7α,12α-trihydroxycoprostanic acid and which may prove to be due to the 5α-epimer of this substance. If allo bile salts are primary (i.e. made in the liver from cholesterol) in these animals, it is by no means clear why so many lizard groups have apparently evolved with substances of this type. The ancestral amphibian forms probably had allo alcohols, such as 5α-cyprinol or 5α-bufol, shortening of the side-chain of which occurred without the development of enzymes stereospecific for reduction leading to the 5β-configuration. Thus, we can trace allo bile salts from *Latimeria* and lungfish through salamanders and certain frogs to lizards, but most vertebrate groups whose ancestors might be presumed to have once had bile salts of this type have evolved to have C_{27} or C_{24} 5β-acids.

One group (Diploglossa) of lizards, at least, is exceptional, for members of the family Varanidae (monitors) and also one of the species of *Heloderma* (the beaded poisonous lizards of Central America) have, as chief bile salts, taurine-conjugated C_{27} acids that are almost

certainly epimers (at C-24 or C-25) of 3α,7α,12α,24ξ-tetrahydroxy-5β-cholestan-26(or 27)-oic acid (*10*), (Fig. 1). The substance obtained from the bile of monitors (varanic acid) is spectroscopically and chromatographically indistinguishable (55) from a stereochemically mixed, partially synthetic sample of *10* (56), and the *Heloderma* acid behaves like an isomer of varanic acid. An isomer of the acid *10* is converted to cholic acid by rat liver mitochondria or 105,000 *g* supernate fortified with NAD^+ or $NADP^+$ and is made from the acid *9* by the mitochondrial or mitochondrial and supernatant fractions (57). In guinea pigs with bile fistulae, tritium-labeled *10* or stereoisomers, injected intraperitoneally, formed cholic acid in a yield of 25% as measured by recovered radioactivity (58). If *10* is generally a normal biosynthetic intermediate, then it can be said that lizards that have a substance of this structure as a chief bile acid perhaps have progressed as far as *10* in bile salt evolution but not further than this, although taurocholate may also be present in *Varanus*. It is still necessary to elucidate the stereochemistry at C-24 and C-25; this is especially difficult, since β-hydroxy carboxylic acids are easily dehydrated on heating in the alkaline conditions necessary to hydrolyze taurine conjugates. If, however, it is the case that *Varanus* and *Heloderma* are using different C-24/C-25 epimers of *10*, there exists an intriguing case for investigation on biochemical evolutionary lines.

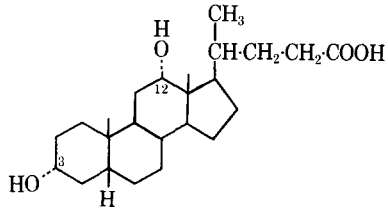
The snakes descended from lizards of some kind; there is no fully convincing evidence of the nature of the limbed precursors (59). It might be hoped that bile salt biochemistry would be helpful here, but this does not seem likely. The many snakes whose bile salts have been examined fall into three groups, having cholic acid, pythocholic acid, or C-23 hydroxylated 5β-cholanoic acids as their principal bile acids. All these C_{24} acids are of the 5β-configuration and thus either the snakes descended from lizards (such as ancestral monitors) that had 5β,C_{27} acids, or evolution to 5β,C_{24} acids occurred in forms that had allocholic acid, of which there is now only traces in snake bile. (It is assumed that evolution from 5α- to 5β-bile salts could occur, but not the reverse). If the latter is the case, i.e., if allocholic was replaced by cholic acid, there must have been something about the circumstances of ancestral snakes that was missing from those of the lizards now having allocholic acid; it is perhaps more plausible that ancestral snakes already had 5β,C_{27} bile acids.

The most primitive snakes examined, of the genus *Typhlops*, have taurine-conjugated cholic acid, but boas and pythons have been found (24)—with one exception (*Corallus enhydris*)—to contain the taurine conjugate of pythocholic (3α,12α,16α-trihydroxy-5β-cholanoic) acid (*33*) as a principal bile salt.



33. Pythocholic acid

When the chemistry of pythocholic acid had been elucidated it presented a challenge to Bergström and his colleagues, who had formulated the view that a first step in biosynthesis was hydroxylation of cholesterol at C-7α. Studies with a python having a biliary fistula showed (9) that the biosynthetic precursor of *33* was deoxycholic (3α,12α-dihydroxy-5β-cholanoic) acid (*34*) or its taurine conjugate.



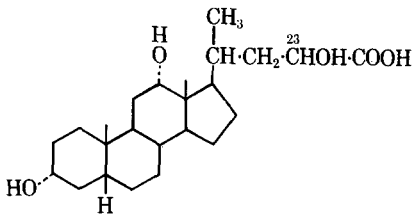
34. Deoxycholic acid

Thus the distinguishing feature of boid snakes is the possession of liver enzymes capable of 16α-hydroxylation of deoxycholic acid. Deoxycholic acid is apparently always made by intestinal microorganisms from cholic acid, so that these snakes are responding to the effect of enterohepatic changes, as do rats and mice which have liver enzymes that catalyze 7α-hydroxylation of deoxycholic acid (60). The boid *Corallus enhydris* which does not apparently contain pythocholic acid, may have 7α-hydroxylating enzymes, as may other snakes with taurocholate as the chief bile salt, or it may lack an intestinal flora that is capable of deoxygenation of cholic acid.

Primitive snakes of the genus *Cylindrophis* probably also have pythocholic acid but it has not been detected in any other group.

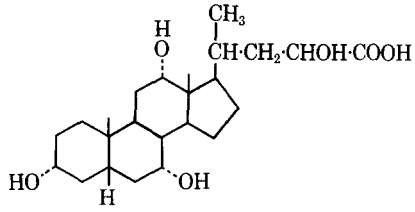
Snakes with C-23-hydroxylated bile acids have been found in all members examined of the genera *Enhydris*, *Helicops*, *Homalopsis*, and *Pseudoboa* of the family Colubridae, and *Atheris*, *Bitis*, *Eristocophis*, and *Vipera* of the subfamily Viperinae; they have not so far been detected in the families Acrochordidae and Elapidae or the subfamily Crotalinae (family Viperidae) (24). In the puff-adder *Bitis arietans* and the Gaboon viper *B. gabonica* the principal bile salt was the taurine conjugate of bitocholic (3α,12α,23ξ-trihydroxy-5β-cholanoic) acid (24).

It may be presumed that this acid (*35*), lacking a 7α-OH group, is the result of 23-hydroxylation of deoxycholic



35. Bitocholic acid

acid (*34*) and, if this is so, the minor amounts of 3α,7α 12α,23ξ-tetrahydroxy-5β-cholanoic acid (*36*) found in *Bitis* bile [and also in *Vipera russelli* (24)] may be formed by the action of the same enzymes on cholic acid.



36. 3α,7α,12α,23ξ-Tetrahydroxy-5β-cholanoic acid

In this view, *36* is a sort of biochemical accident and it will be of interest to see if bitocholic acid is also the principal bile acid in the other snakes in which the taurine conjugate of *36* can be detected chromatographically.

Many other snakes have been found to have taurocholate as almost the only bile salt (24); deoxycholic acid was detected also (61) in *Trimeresurus flavoviridis* (Crotalinae).

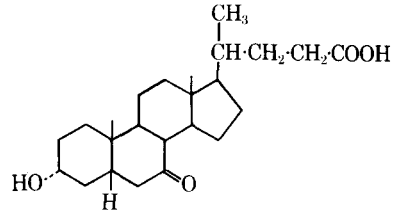
The bile of the archosaurs (crocodiles and alligators) contains C_{27} acids, of which 3α,7α,12α-trihydroxycoprostanic acids (C-25 epimers of *9*, Fig. 1) are the principal constituents. Small amounts of 3α,7α-dihydroxycoprostanic acid have been found in *Alligator mississipiensis* (62). An alligator with a bile fistula made radioactive 3α,7α,12α-trihydroxycoprostanic acid (probably the isomer *9*) from cholesterol-4-^{14}C, but cholic acid was not found (63). Crocodilians may be said to have evolved to stage *9* in the biosynthetic scheme (Fig. 1) but not further. Phenotypically, these reptiles are an extremely conservative group and if they are called evolutionarily "senescent," their bile salts seem to present also this type of biochemical picture.

BIRDS

The bile salts of birds are not at all well-known as only a few species have been thoroughly examined. No C_{27} bile acids have been reported and the chief C_{24} acids (conjugated entirely with taurine) seem to be cholic and chenodeoxycholic, as in mammals. It was at one time thought that chenodeoxycholic acid (from the Greek, $\chi\eta\nu$ a goose) was characteristic of bird bile but this idea was based on knowledge of the bile of the largely vegetarian domestic fowl and goose, which contain only minor amounts of cholic acid.

The carnivorous penguins proved to have about as much cholic as chenodeoxycholic acid (24), and allocholic acid was easily found. A similar bile acid mixture may occur in other flesh-eating birds; it may well turn out that dihydroxy- or trihydroxy-type bile acids are correlated with vegetarian or carnivorous dietary habits respectively, as in mammals.

Bird bile contains a number of unidentified bile acids; some of these may prove to be artifacts of the enterohepatic circulation (secondary bile acids) like deoxycholic acid (*34*) and the 3α-hydroxy-7-oxo-5β-cholanoic acid (*37*) that occurs in the bile of conventional but not in that of germ-free chicks (64).



37. 3α-Hydroxy-7-oxo-5β-cholanoic acid

There seems to be nothing in the bile of two "ratite" (flightless) birds examined, the emu and rhea, to suggest any fundamental bile salt difference between these and the remaining ("carinate") birds.

MAMMALS

The mammals are divided into Protheria and Metatheria, and the surviving Protheria include three species of egg-laying mammals, the monotremes, whose evolutionary origins have long been a matter for discussion (65).

The bile of two of the monotremes, the Australian duck-billed platypus (*Ornithorhyncus anatinus*) and spiny anteater (*Tachyglossus aculeatus*) contained cholic, chenodeoxycholic, and probably deoxycholic acid, all taurine-conjugated; no bile alcohols or C_{27} acids were found (66). Monotreme bile salts thus seem unhelpful to those interested in the origins of these mammals.

The Metatheria comprise the marsupials of Australia and America and are a group occupying many of the available ecological niches. Only taurine conjugates have been found in these animals, although some examined have an exclusively vegetarian diet. An example is the koala *Phascolarctos cinereus*, whose bile salts contain little but taurine-conjugated 3α-hydroxy-7-oxo-5β-cholanoic acid, *37*. It is not known whether this acid is a secondary product in the koala; if it is, then chenodeoxycholic acid (*15*), found in traces in the bile (24), is probably the primary acid made in the liver from

cholesterol. The other marsupials examined (an opossum and two kangaroo species) (24) have a rather more varied diet and cholic, chenodeoxycholic, and deoxycholic (*34*) acids, as taurine conjugates, comprise most of the bile salts. It is doubtful whether glycine conjugates occur in the marsupials whose bile has been scrutinized; if they do, it is in very small amounts.

None of the very few marsupial carnivores has been examined; it would be interesting to discover, for example, whether the Tasmanian marsupial wolf *Thylacinus* shares with placental dogs their almost complete adherence to taurocholate as a bile salt.

Bile of the placental (eutherian) mammals, which, like the birds, do not all have gallbladders, has been extensively examined. Representatives of all the living Orders listed by Simpson (67) except the Dermoptera ("flying lemurs"), Chiroptera (bats), Hyracoidea (hydraxes), and Sirenia (dugongs and manatees) have received at least a superficial investigation and in the case of some domestic and laboratory animals, including man, it seems that almost everything is known about the chemistry of the bile salts in health.

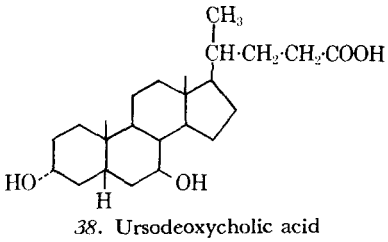
Glycine conjugation, in easily detectable degree, is found only in eutherians; thus the enzyme system that catalyzes the reaction

$$\underset{\text{Bile acid CoA}}{R.CO.S.CoA} + \underset{\text{Glycine}}{H_2N.CH_2.COOH} \rightarrow \underset{\text{Glycine conjugate}}{R.CO.NH.CH_2.COOH} + CoASH$$

is present. It is interesting to speculate whether this system arose by evolution more than once; if it did not, the distribution of glycine conjugates might be of interest to students of eutherian mammalian radiation.

It has never been shown convincingly that glycine conjugates have advantages over taurine conjugates for any of the physiological functions attributed to bile salts; it has, however, been suggested that they might conceivably play some part in the control of certain intestinal parasites (68).

Except for certain "unique" bile acids, discussed later, the chief eutherian primary bile acids are cholic and chenodeoxycholic (*15*), with 3α-hydroxy-7-oxo-5β-cholanoic (*37*) and ursodeoxycholic [3α,7β-dihydroxy-5β-cholanoic (*38*)] acids present in fair proportion in some species.

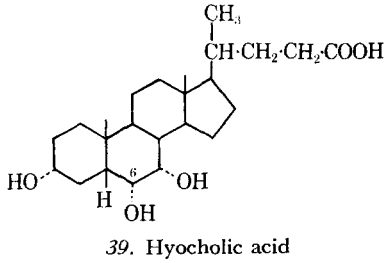


38. Ursodeoxycholic acid

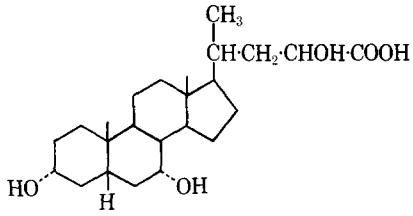
The acids *37* and *38* can probably sometimes be primary bile acids, but can also be formed from chenodeoxycholic acid by intestinal microorganisms. Deoxycholic acid (*34*) is a common constituent of mammalian bile.

There is an obvious association between mammalian bile salt type and diet. Carnivores have taurine conjugates of cholic or other trihydroxycholanoic acids, herbivores generally have dihydroxy or ketohydroxy acids with, frequently, a high proportion of glycine conjugates, and omnivores have a good proportion of these various types. Bovids are an exception to this generalization, for these herbivores seem to have bile salts of the "omnivorous" type, rich in cholic acid and taurine conjugates as well as in chenodeoxycholic and deoxycholic acids and conjugates of glycine. The omnivorous laboratory rats and mice (Murinae) have taurocholate as the chief bile salt and possess liver enzymes that rehydroxylate deoxycholic acid at C-7α to re-form cholic acid (60), which suggests that these rodents, like carnivores, "prefer" trihydroxy bile acids. No such enzymes have been found in man or, indeed, in any other animal form, including the eel *Anguilla japonica* (69). Deoxycholic acid is generally allowed to persist in the bile, and in the laboratory rabbit *Oryctolagus cuniculus* its glycine conjugate comprises almost all the bile salts. Thus, this animal seems to rely on its intestinal microflora to maintain the dihydroxy type of bile salts that other examples suggest to be advantageous to vegetarian mammals. Its capacity to make chenodeoxycholic acid is so small that glycochenodeoxycholate has but recently been detected in the bile (70) in spite of previous very careful searches; the amout of taurine conjugation is small.

A number of "unique" cholanoic acids have been found in mammalian bile (24): the certainly or probably primary acids are hyocholic (3α,6α,7α-trihydroxy-5β-cholanoic, *39*) in pigs (Suidae) and probably in the wart-hog *Phacochoerus aethiopicus;* α- and β-muricholic (3α,6β,7α- and 3α,6β,7β-trihydroxy-5β-cholanoic, *16* and *16a*) in Murinae; phocaecholic (3α,7α,23ξ-trihydroxy-5β-cholanoic, *40*) in Pinnipedia (seals, sea lions, and walruses); and 3α,7α,12α,23ξ-tetrahydroxy-5β-cholanoic acid, *36*, also in Pinnipedia.

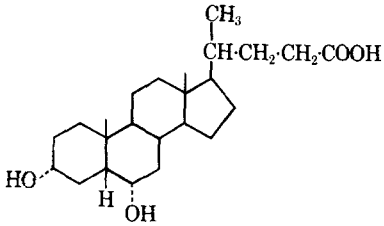


39. Hyocholic acid



40. Phocaecholic acid

It will be noticed that all these acids, with the exception of *16a* and *36*, can be considered to be hydroxylated chenodeoxycholic acids; it has, indeed, been shown that hyocholic and α-muricholic acids can be made in vivo from chenodeoxycholic acid (60) and that α-muricholic acid is a precursor of *16a* (71). It therefore seems a reasonable speculation that the ancestors of at least some of the animals now making " unique" bile acids needed, because of some change in their dietary circumstances, to make increased amounts of trihydroxy bile salts and found it biochemically more expedient to do this by developing enzymes capable of hydroxylating chenodeoxycholic acid than by increasing cholic acid biosynthesis. This might imply that the ancestors of the Pinnipedia, for example, were more herbivorous than the pinnipeds now are, a deduction that could be tested by fossil evidence. On the other hand, it is possible that very small proportions of unique cholanoic acids, such as α- and β-muricholic acids, arise by a biochemical "accident" and thus have no gross historical significance. The pig (*Sus*), now omnivorous, presents a curious picture. Its ancestors probably made cholic acid, which has been found in traces in the bile (72, 73), as the related wart-hog now does (24), but at present pig bile salts are chiefly a mixture of hyocholic, hyodeoxycholic (3α,6α-dihydroxy-5β-cholanoic, *41*), chenodeoxycholic, and 3α-hydroxy-6-oxo-5α or 5β-cholanoic acids, conjugated principally with glycine.



41. Hyodeoxycholic acid

All these acids except hyocholic and chenodeoxycholic are secondary (60): as mentioned above, chenodeoxycholic can be hydroxylated to hyocholic. The suids, therefore, have a biosynthetic balance between hyocholic and chenodeoxycholic as most mammals do between cholic and chenodeoxycholic acids; the control in suids, however, concerns the enzymic system for hydroxylating chenodeoxycholic acid. It does, indeed, seem that in pigs a 6α-hydroxylase with chenodeoxycholic acid (or its conjugates) as a substrate was preferred during evolution to a reactivation of cholic acid biosynthesis.

The traces of hyodeoxycholic acid in rat bile are secondary and are the result of quite complex reactions, some caused by intestinal microflora and others by liver enzymes (74, 75).

There is room for further work on the possible taxonomic significance of mammalian bile salt differences. It is perhaps too much to hope for the discovery of a C-23-hydroxylated cholanoic acid in an existing terrestrial mammal that would throw light on pinniped history, but a further exploration of the few pig genera might well be taxonomically helpful. The New World pig (the peccary) has no gallbladder so that fistula bile would probably be required for bile salt examination, which might possibly yield results of great interest.

The Sirenia (dugongs and manatees) have not been examined; it is conceivable that bile salt studies could throw light on their, at present obscure, history.

A taxonomic exercise in rodents was a comparison (76) between the South American coypu *Myocastor coypus* and the West African cutting-grass *Thyronomys swindereanus*, quite similar rodents placed by Simpson (67) in the same superfamily (Octodontoidea). It was found that whereas coypu bile salts consisted of chenodeoxycholic, ursodeoxycholic, and 3α-hydroxy-7-oxo-5β-cholanoic acids with a high proportion of glycine conjugates, those of *Thyronomys* contained chiefly taurochenodeoxycholate; glycine conjugates could not be detected. The conclusion was against a very close relationship between these rodents; it could be supposed that they did not have a glycine-conjugating common ancestor, although the proportion of glycine conjugates in rats and men can be made to vary by hormonal or dietary stresses and alters in disease (24).

Do the various New World rabbits share the adherence of *Oryctolagus* to deoxycholic acid and glycine conjugates; do the "native" mice of America have the muricholic acids; is there a general tendency to glycine conjugation in South American rodents (as in the guinea pig and coypu)? These and many more questions about mammalian bile salts remain to be answered.

GENERAL CONSIDERATIONS

There can be little doubt that bile salts have evolved and it seems reasonable to suppose that the progression has been from those alcohols nearest cholesterol to cholic, chenodeoxycholic, and related C_{24} cholanoic acids. It would be difficult to explain the association between primitive animals and (presumably) biochemi-

cally less advanced bile salts on any other hypothesis. If this evolutionary course is accepted, does it mean, as is generally assumed, that fresh DNA base-sequences, capable of coding for the synthesis of new enzymes, arose by mutation as bile salt evolution progressed, or could there possibly be some other mechanism? Could, for example, the DNA base-sequences have existed a priori in some ancestral vertebrate and the evolutionary processes have consisted in the development of methods of bringing them into activity? Such ideas seem strange or untenable at present, but so little is understood about the control of biochemical evolution at the molecular level that an open mind must be kept.

A curious feature about bile salt distribution is the occurrence in some animal forms of small amounts of more advanced bile salts, although the principal substances are primitive. Examples are 5α-cyprinol in *Latimeria*, cholic acid in cyprinids [shown to be formed in *Carassius carassius* after intraperitoneal injection of cholesterol-4-^{14}C (77)], ranids, and possibly elasmobranchs, and allocholic acid in salamanders. It is hard to see how the possession of these bile salts, in traces, could confer a selective advantage sufficient to ensure their survival and (presumably) eventual increase in evolving forms. To say that development of advanced bile salts is bound up with other progressive changes in the phenotype is uninformative and dissociates bile salt evolution from selective pressures.

It is also remarkable that in every major vertebrate group, except possibly chelonians, the progression seems to be to C_{24} acids. It is hard to escape the conclusion that cholic acid itself has been produced by evolution at least twice, once in teleosts and once in tetrapods. Why, if mutations produce new enzymes, should this directionality be maintained? Perhaps the initial selection in all groups, except myxinids, of the 3,7,12 pattern of hydroxylation implied that allocholic, cholic, and chenodeoxycholic acids were the only possible C_{24} acids that could ensue; as we have seen, other C_{24} acids could have arisen from these.

Finally, in some groups, bufonids and archosaurs for example, there is little sign that cholic acid can be made. Does this mean that these animals are "senescent" in a biochemical as well as in a morphological evolutionary sense? Such a condition could be contrasted with evolutionary "vigor" in, say, ranids.

Clearly important future biochemical tasks, now that the chemistry and distribution of bile salts are reasonably well known, will be to explore biosynthetic pathways in lower groups in order to discover how general the scheme in Fig. 1 is in vertebrates and to attempt to elucidate the situation at enzymic and nucleic acid levels in different animal forms, in an endeavor to understand more fully the nature of the evolutionary process.

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