Comparative Studies of 'Bile Salts'

4. BILE SALTS OF THE EUROPEAN FROG, *RANA TEMPORARIA*

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Previous studies of the bile salts of frogs and toads have been carried out on two species only, and entirely by Japanese workers. Okamura (1928, 1929) isolated 'bufocholane', m.p. 236°, and an acid described as 'bufodeoxycholic acid' from the winter bile of the toad, *Bufo vulgaris* (Jap.). Taurine was also separated from the bile. Makino (1933) obtained from the bile of the same species an alcohol, m.p. 230°, described as tetrahydroxybufostane, $C_{27}H_{48}O_4$, which showed positive Pettenkofer and Hammarsten tests. Trihydroxybufosterocholenic acid, $C_{28}H_{46}O_5$, was described by Shimizu & Oda (1934) as occurring in the toad bile and this was converted by Shimizu & Kazuno (1936*b*) to bisnorcholic acid; it was thus shown to belong to the cholane series and to have in its molecule three hydroxyl groups in the same position as those in cholic acid. Trihydroxy*iso*sterocholenic acid, $C_{28}H_{46}O_5$, was described by Shimizu & Kazuno (1936*a*) and this was also converted by Shimizu & Kazuno (1937) to bisnorcholic acid. Kazuno (1940) published an account of a careful and detailed study of that part of the (acidified) bile of the Japanese toad from which the above-mentioned acids, apparently occurring in the unconjugated form, had been removed by ether extraction. The chief constituent was a sulphate, m.p. 197°, which by alkaline hydrolysis yielded pentahydroxybufostane, $C_{28}H_{50}O_5$, m.p. 172°. About 10 g. of this substance were isolated from 4000 gall-bladders (5 l. of bile), in one, and 20 g. from 8500 toads (10 l. of bile) in a second experiment. Pentahydroxybufostane was extensively investigated and its triacetate, m.p. 119°, converted in a yield of about 10 % to cholic acid, which was identified as the acid itself and as its ethyl ester. However, as in the case of the acids, conclusive proof of the C_{28} formula was not given, and it rests only on analytical figures. A neutral compound apparently identical with the alcohols of Okamura (1928) and Makino (1933) was also isolated after alkaline hydrolysis of bile fractions and was renamed tetrahydroxynorbufostane, on the assumption that its molecule contained 27 carbon atoms. A third neutral substance, m.p. 175°, was stated to occur in the unconjugated state in the bile: it was called tetrahydroxycholane, and assigned a C_{24} formula. Like pentahydroxybufostane, it gave positive Pettenkofer and Hammarsten reactions, indicating OH groups probably at $C_{(3)}$, $C_{(7)}$ and $C_{(12)}$ in the steroid nucleus.

Bile of the frog *Rana catesbiana* was examined by Kurauti & Kazuno (1939), who isolated cholesterol, an acid (named trihydroxybisnorsterocholanic acid) giving a negative response in the Hammarsten reaction and also the sulphate, m.p. 178°, of a so-called tetrahydric alcohol. By alkaline hydrolysis of this latter substance there was obtained an unsaturated alcohol of m.p. 177°, which was quite arbitrarily given a C_{24} formula and named trihydroxycholene. The acetate (? diacetate) had m.p. 180°. Mabuti (1941) investigated the trihydroxybisnorsterocholanic acid of Kurauti & Kazuno and concluded that it also had OH groups at $C_{(3)}$, $C_{(7)}$ and $C_{(12)}$ as in cholic acid.

The above work showed that further examination of amphibian bile was likely to yield results of much interest and the present report is of a preliminary investigation of the bile salts of *R. temporaria*.

RESULTS

An interpretation of the experimental findings is given in Fig. 1. The chief constituent of the bile was a sulphate ester, readily obtained as the sodium salt (I) as previously described (Haslewood & Wootton, 1950). If the (probably) pentahydric alcohol with which the sulphate is esterified is called 'ranol' and the isolated bile salt 'sodium ranol sulphate', the chief product of acid hydrolysis, as carried out, appeared to be ranol (II) itself, not obtained crystalline, but easily converted by mild acetylation in fair yield into a tetraacetate (IV), which could be oxidized to a monoketone (V). Alkaline hydrolysis of ranol sulphate, on the other hand, led to a mixture from which it was not, except in one experiment, possible to isolate the above tetraacetate. However, there was obtained after partial acetylation a mixture of crystalline acetates, the analysis of one of which (VI) suggested that the main product of alkaline hydrolysis, as usually carried out, was a substance derived from ranol sulphate by the elimination of an OH group, together with the

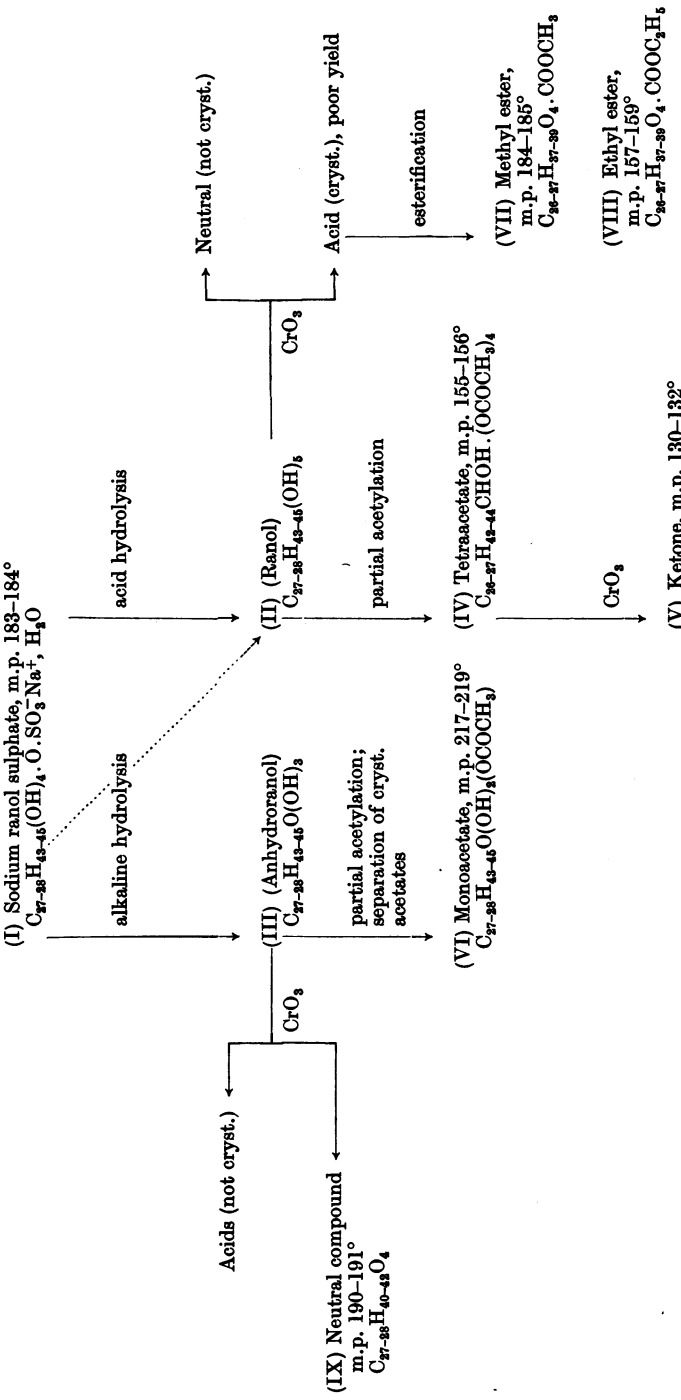
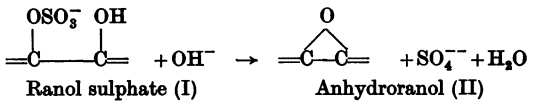


Fig. 1. Compounds derived from the bile of *Rana temporaria*. (Formulae on the assumption that ranol is a steroid whose molecule contains 27 or 28 carbon atoms.)

sulphate. Since the acetate (VI) appeared to be saturated, the chief product (not crystallized) formed by the above reaction most probably contained an oxide ring and is referred to tentatively as 'anhydroranol' (III), e.g.



Ranol sulphate (I) Anhydroranol (II)

The product of alkaline hydrolysis could also be converted by oxidation into a mixture from which was readily isolated a neutral substance (IX), whose probable formula can be explained on the assumption that a —CH_2OH group in anhydroranol (III) was oxidized to —CHO and two =CHOH groups to =C:O, the oxide ring remaining unaffected.

A crystalline acid, purified as the methyl and ethyl esters (VII and VIII) was obtained by chromic oxidation of a hydrolysed mixture from which the only crystalline material isolated by acetylation was the tetraacetate (IV). It is tempting to assume that this acid arose by oxidation of a —CH_2OH group in ranol to —COOH and the conversion of four =CHOH groups to =C:O, an interpretation which is supported by the analytical figures and which is shown in Fig. 1. However, the acid was obtained in very small yield and its preparation often failed, so that some doubt is felt as to its really arising in the manner suggested. The above esters were compared with, and found to be different from, the appropriate esters derived from dehydronor- and dehydrobisnor-cholic acid, prepared from cholic acid as described by Shimizu & Kazuno (1936*b*).

EXPERIMENTAL

General. All melting points are uncorrected. Optical rotations were determined in a 1 dm. microtube; microanalyses were done by Weiler and Strauss, Oxford, and by Mr A. T. Macdonald, Edinburgh. Al_2O_3 was supplied by Hopkins and Williams Ltd.; l.p. = light petroleum, b.p. 40–60°. 20 % CrO_3 was as described by Haslewood & Wootton (1951). H-test = Hammarsten's HCl test (Haslewood, 1943).

Substances derived from the bile of Rana temporaria

Sodium ranol sulphate (simplified preparation). A sample, m.p. 183–184°, was prepared and analysed by Haslewood & Wootton (1950). A simpler preparation was as follows: 392 gall bladders, preserved in ethanol, were ground in a mortar and thoroughly extracted with fresh ethanol. The filtered extract was evaporated and the residue extracted several times with ethanol in an evaporating dish at room temperature. The ethanol-insoluble residue was collected, washed with ethanol and dried *in vacuo* over H_2SO_4. It was a light-brown microcrystalline powder of m.p. 185° (gas), and weighed 1·23 g. A further crop was obtained by evaporation of the ethanol extracts and washings, followed by re-extraction of the residue with cold ethanol: the ethanol-insoluble material weighed 0·23 g. and had m.p. 182° (gas).

Acid hydrolysis of ranol sulphate. Sodium ranol sulphate (0·5 g.) was dissolved in 0·25 N-HCl (20 ml.) and the solution in a flask was heated, with occasional shaking, in a gently boiling-water bath. After 1–2 hr. a gel formed and the amount of this slowly increased. After about 20 hr. heating the gel was collected on a filter, squeezed as far as possible free from aqueous acid, washed with water and dried *in vacuo* over H_2SO_4. Yield, 0·35 g. (approx. 89 %, calculated on the proposed formulae) of a brown solid, giving a blue colour in the H-test. This is regarded as mainly crude ranol (II); it could not be crystallized.

Ranol tetraacetate (IV). The above material (0·1 g.) was dissolved in dry pyridine (1 ml.) with acetic anhydride (1 ml.) and the mixture left at about 20° for 19 hr. with occasional shaking. Dilution with aqueous HCl precipitated a brown gum which was collected after about 4 days and dissolved in ethanol. The filtered solution was evaporated and the residue crystallized from an approx. 30 % (v/v) mixture of ether/l.p. The crude acetate (80 mg.) was then recrystallized from l.p./benzene from which it gave white needles (47 mg.) of m.p. 146–148°. Crystallization from dilute methanol and elution from Al_2O_3 with benzene, followed by further recrystallizations from l.p./benzene finally gave white needles of *ranol tetraacetate* (IV) which had m.p. 155–156°. $[\alpha]_D^{23°} = -12 \pm 4°$ in $CHCl_3$ (*c*, 1·0). (Found: C, 68·0, 67·8; H, 9·4, 9·4. $C_{35-36}H_{56-58}O_9$ requires C, 67·7–68·1; H, 9·0–9·2 %.)

A sample of this acetate (15 mg.) was made from the product (24 mg.) of the acid hydrolysis of the purified bile salt (50 mg.) described by Haslewood & Wootton (1950).

Monoketone (V). The above acetate (47 mg.) in acetic acid (0·5 ml.) was treated with 20 % CrO_3 (0·05 ml.), together with water (1 drop), to dissolve the precipitate. After 10 min. the solution was diluted and the product which separated on standing collected, washed with water and dissolved in ethanol. Evaporation of the ethanol left a residue which crystallized from l.p. and the l.p./benzene in white needles. This *ketone* (V) had m.p. 130–132°. (Found: C, 67·8; H, 8·2. $C_{35-36}H_{54-56}O_9$ requires C, 68·0–68·4; H, 8·7–8·9 %.) $[\alpha]_D^{24°} = +27 \pm 2°$ in $CHCl_3$ (*c*, 0·5).) The *semicarbazone*, prepared in the usual way, was a partially crystalline powder of m.p. 171–175°. (Found: N, 6·1. $C_{36-37}H_{57-59}O_9N_3$ requires N, 6·2–6·1 %.)

Esters (VII) and (VIII). The only samples of the acid giving these esters were prepared from a product made by alkaline hydrolysis of the bile salts under conditions not very accurately defined, but which included heating crude bile salts (*c*, 5 g.) for about 21 hr. in NaOH solution (30 g./l.). The neutral product which separated was collected, washed and dried; it gave on partial acetylation the acetate (IV) as the sole crystalline material isolated. A similar alkali-hydrolysed product could not be prepared again, under more carefully defined conditions of hydrolysis, for all other neutral substances obtained from the bile salts by alkali treatment gave derivatives apparently of the anhydroranol (see below). Several oxidations of the above product were carried out; the following experiment was one of those which led to crystalline material: the above-mentioned alkali-hydrolysed product (0·2 g.) in acetic acid (2 ml.) was treated, with cooling to about 20°, with 20 % CrO_3 (2 ml.) added gradually with shaking. After 2 hr. the solution was diluted and treated with NaCl (excess). The gummy precipitate was collected after about 16 hr.; it was washed with water and

then boiled with N-NaOH. The cooled mixture was filtered from gummy neutral material and the filtrate acidified with H_2SO_4. After the addition of NaCl (excess) the precipitated acid was collected, washed with water and dried *in vacuo* over H_2SO_4. Yield: 80 mg. of acidic material which, from dilute ethanol, gave needles (25 mg.) of m.p. approx. 211–218° (decomp.). These were gently boiled for 1 hr. under reflux with 2·5 ml. of a mixture of ethanol (5 ml.) and H_2SO_4 (0·5 ml.). The cooled solution was diluted with Na_2CO_3 solution (excess) and the insoluble material collected, washed and crystallized from dilute ethanol, from which the *ethyl ester* (VIII) formed leaflets (10 mg.) of m.p. 157–159°. (Found: C, 71·6, H, 8·7. $C_{29-30}H_{42-44}O_6$ requires C, 71·6–72·0; H, 8·6–8·8%.)

Another sample (25 mg.) of the above crude acid was esterified with diazomethane. The product, in ether, was washed with dilute NH_3 solution and water, and the solvent was removed. The residue, in benzene, was eluted from an Al_2O_3 column with benzene and crystallized from l.p./benzene, from which the *methyl ester* (VII) gave short white needles of m.p. 184–185°. (Found: C, 71·5; H, 8·1. $C_{28-29}H_{40-42}O_6$ requires C, 71·2–71·6; H, 8·5–8·6%.)

Alkaline hydrolysis of ranol sulphate (leading to anhydroranol derivatives). Except in the case given above, it did not prove possible to prepare the same derivatives from the alkali-hydrolysed bile salts as could be made from the material derived by acid. An effective method of alkaline hydrolysis was as follows: a solution of the bile salts (0·2 g., purified and of m.p. 183–184°) in water (4 ml.) with 5N-NaOH (1 ml.) was sealed in a metal bomb and heated at about 110° for 8 hr. The bomb was cooled and its contents washed out with water. The precipitated solid was collected, washed with water and dried *in vacuo* over H_2SO_4. Yield: 0·14 g. (approx. 92%, calculated on the suggested formulae) of a light brown powder, giving a feeble response in the H-test. By addition of $BaCl_2$ to the acidified liquors, it was found that the sulphate content (as S) of the bile salts was about 5·9%.

The acetate (VI). The above product (0·13 g.) in pyridine (1 ml.) was treated with acetic anhydride (1 ml.) and the mixture was warmed to about 50° and then left at about 22° for 16 hr. The solution was diluted with water and 5N-HCl and extracted with ether. The ether was washed with water, NH_3 solution, water, dried (Na_2SO_4) and evaporated. The residue readily crystallized from l.p./ether, giving crystals (70 mg.) of m.p. 175–204°. This material was recrystallized from dilute ethanol after which the product (40 mg.) had m.p. 202–216°; this mixture was then eluted from a column containing Al_2O_3 (0·5 g.) with benzene (40 ml.). After three recrystallizations of the eluted material (30 mg.) from dilute ethanol, the *acetate* (VI) formed glistening white leaflets of m.p. 217–219°. (Found: C, 72·9, 73·3; H, 9·6, 9·7. $C_{29-30}H_{48-50}O_5$ requires C, 73·1–73·5; H, 10·1–10·2%.)

Alkaline hydrolysis of this acetate in the usual way gave a gelatinous solid which, after drying, responded feebly, if at all, to the H-test. On the other hand, the acetate (IV) (see above) gave on hydrolysis a gel, which, after drying, gave a definite purplish colour in this test.

The neutral compound (IX). The above-described material (0·1 g.), made by bomb hydrolysis with alkali, was dissolved in acetic acid (1 ml.) and treated at 19° with 20% CrO_3 (1 ml.) added gradually with mixing. After 1·2 hr. at 20°, the solution was diluted and saturated with NaCl. The precipitated solid was collected, washed with water and stirred with warm 0·1N-NaOH. The insoluble material was filtered off. (The filtrate gave an amorphous acidic mixture (19 mg.) on acidification.) The alkali-insoluble precipitate was dissolved in ethanol and the filtered solution evaporated. The residue (23 mg.) was a partially crystalline solid of m.p. 173–179°. This was eluted from Al_2O_3 (0·4 g.) with benzene (15 ml.) and recrystallized from ether, from which it formed white needles. Thus prepared, the *neutral compound* (IX) had m.p. 189–190·5°. (Found: C, 76·4, 76·6; H, 9·2, 9·1. $C_{27-28}H_{40-42}O_4$ requires C, 75·7–76·0; H, 9·4–9·5%.)

Derivatives of nor- and bisnor-cholic acid

Nor- and bisnor-cholic acids were prepared as described by Shimizu & Kazuno (1936*b*). Prepared in the usual way, *ethyl dehydronorcholate* crystallized from dilute ethanol as long white needles of m.p. 234–235° (decomp.). (Found: C, 71·4, 71·4; H, 9·0, 8·8. $C_{25}H_{36}O_5$ requires C, 72·1; H, 8·7%.)

Bisnorcholic acid was esterified with diazomethane and the product oxidized with CrO_3 in the usual way. The crude dehydro ester was purified by elution from Al_2O_3 with benzene and crystallized from l.p./benzene, from which *methyl dehydrobisnorcholate* formed long white needles of m.p. 191–193°, depressed by the methyl ester (VII). (Found: C, 70·9; H, 8·4. $C_{23}H_{32}O_5$ requires C, 71·1; H, 8·3%.)

DISCUSSION

Biological. The reported work agrees with that of the Japanese on *R. catesbiana* and on the Japanese toad, in that the main bile salt of *R. temporaria* also appears to be a neutral alcoholic substance or substances conjugated with sulphate. The amount of bile used in this research was insufficient for the identification of the minor quantities of acids which were also present.

The 'sodium ranol sulphate' now investigated appears to resemble closely, if not to be identical with, the sulphate, m.p. 178°, isolated by Kurauti & Kazuno (1939) from *R. catesbiana*, but a careful scrutiny of Kazuno's (1940) report on pentahydroxybufostane and other neutral substances from toad bile has failed to suggest the identity of these or any of their derivatives with material from *R. temporaria*. The conclusion is that at least two species of *Rana* probably contain the same bile salt, but that this is different from similar compounds in the bile of one species of *Bufo*.

Okasaki (1944) has reported that the chief bile salt of the aquatic salamander *Diemyctylus phyrrhogaster* is likewise the sulphate ester of a neutral steroid alcohol.

The presence of such neutral substances in bile of amphibia would suggest, on the hypotheses put forward by Haslewood & Wootton (1950), that these creatures are evolutionarily of a primitive type and that they agree with at least some of the elasmobranch fishes and with the carp *Cyrinus carpio* (Haslewood, 1951) in this respect. It would be unwise at present to suggest that any closer biological

relationship is indicated by the similarities so far revealed in the chemical nature of the neutral bile salts.

Chemical. The scheme put forward in Fig. 1 to explain the relationships between the crystalline substances isolated must be regarded as tentative. An alternative explanation, that 'sodium ranol sulphate' is a mixture, components of which give rise to the ranol and anhydroranol derivatives, can hardly be upheld since the acetates IV and VI were isolated exclusively after acid and alkaline hydrolysis, respectively, from the same purified sample of the sodium salt. Moreover, although it is difficult to be certain on this point, purified sodium ranol sulphate behaved as a single reproducible compound in melting point and general properties; its analysis agreed fairly well with the postulated formulae. However, the case for the scheme in Fig. 1 would be greatly strengthened if ranol and anhydroranol themselves could be obtained in crystalline form. No trace was detected of the crystalline unsaturated 'trihydroxycholene' of Kurauti & Kazuno (1939), which was presumably derived from their sulphate by elimination of the sulphoxy group, together with an adjacent hydrogen atom, during alkaline hydrolysis.

The amount of significance which should be attached to the feeble or negative response to the Hammarsten test given by anhydroranol is doubtful, for it was found that the carbinols derived by reaction of the Grignard reagent with methyl cholate and methyl norcholate also responded feebly to this test. Clearly, response to a Hammarsten test can be greatly modified by the side chain of the steroid, and although a positive reaction is very probably, in a natural steroid, indicative of hydroxyl groups at $C_{(3)}$, $C_{(7)}$ and $C_{(12)}$, a failure to give a blue or purple colour cannot necessarily be taken as indicating the absence of such groups (see also, for example, Shimizu & Kazuno, 1936*a*, *b*). The colour given by ranol certainly suggests that this substance has three of its hydroxyl groups in the above-mentioned positions. One of the remaining two OH groups in ranol is, possibly, primary, and both were readily acetylated. If the structure suggested (see Results) for compound IX is correct, one at least of the nuclear OH groups must be involved in the formation of the oxide ring in anhydroranol. The monoketone (V) appears to be derived from a steroid containing a free hydroxyl group at $C_{(12)}$ (compare pythocholic lactone, Haslewood & Wootton, 1951).

The analytical figures do not enable one to choose between formulae for a C_{27} or C_{28} steroid for ranol and its derivatives. If ranol is in fact a C_{28} steroid, it would be difficult to explain its derivation from cholesterol, an origin frequently suggested for scymnol and the C_{24} bile acids.

One of the chief objects of this work has been the development of methods which can be applied to other species and might then be expected to lead to the isolation of identifiable crystalline substances. Such a substance, which might be of value in future work, is the so-called ranol tetraacetate (IV).

SUMMARY

1. The chief bile salt found in the frog *Rana temporaria* was a sulphate, now temporarily named 'sodium ranol sulphate', of a pentahydric alcohol, 'ranol', probably $C_{27-28}H_{43-45}(OH)_5$.

Derivatives of this alcohol could be isolated after acid hydrolysis of the sulphate. After alkaline hydrolysis, however, the only crystalline compounds isolated appeared, except in one experiment, to be derived from an 'anhydroranol', a saturated substance whose molecular formula is less by H_2O than that of ranol. It is suggested that the molecule of anhydroranol, like that of scymnol, contains an oxide ring.

2. A relationship between the seven crystalline substances now obtained in purified form from frog's bile is tentatively suggested. It is thought that some of these substances may be of value in future investigations of bile from other species.

3. Ranol may be a steroid whose molecule contains a primary hydroxyl group, with secondary hydroxyl groups at $C_{(3)}$, $C_{(7)}$ and $C_{(12)}$. The remaining OH group was readily acetylated.

4. The possible evolutionary significance of similar sulphate esters in the bile of vertebrates is briefly discussed.

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