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Preparation of the 3-Monosulphates of Cholic Acid, Chenodeoxycholic Acid and Deoxycholic Acid

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1. The 3-sulphates of cholic, chenodeoxycholic and deoxycholic acids were prepared as crystalline disodium salts. 2. The method described shows that it is possible to prepare specific sulphate esters of polyhydroxy bile acids and to remove protecting acyl groups without removing the sulphate. 3. A study of bile acid sulphate solvolysis showed that none of the usual methods give the original bile acid in major yield in a single step. 4. An understanding of the preparation, properties and methods of solvolysis of bile acid sulphates is basic for investigations of cholestasis and liver disease.

It is known that common bile acids and their conjugates may be excreted in human urine as sulphates, especially in cholestasis (e.g. Stiehl, 1972; Makino *et al.*, 1973) and it is therefore of interest to prepare some such sulphates, especially for experiments on solvolysis. For the three chief common bile acids in human bile, namely cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid) and deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid) and their glycine and taurine conjugates, a total of 39 sulphate esters is possible. We describe the preparation and properties of the 3-monosulphates of cholic, chenodeoxycholic and deoxycholic acids.

Palmer & Bolt (1971) described the preparation of the sulphates of lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) and its glycine and taurine conjugates.

Materials and Methods

Where applicable the methods used were those described by Anderson *et al.* (1974). T.l.c. was carried out on glass plates (5cm×20cm) coated with a layer (0.25cm thick) of silica gel G (E. Merck A-G, Darmstadt, Germany) scored lengthwise at about 1mm from each edge to minimize edge effects during running. Plates were activated for 15–30min at 100°C, cooled and loaded. After equilibration (5–10min) the sealed tanks were tilted so that the solvent system ran on to the bottom of the plates. After running, the plates were briefly heated at 100°C, cooled and sprayed with a mixture of phosphomolybdic acid (5g) and acetic acid (100ml) to which H_2SO_4 (7.5ml) was carefully added, with cooling in ice. After stirring at room temperature, this reagent was filtered. It is slightly modified (S. Ikawa, unpublished work) from that of Usui (1963); on heating the sprayed plates at 100°C it gives blue spots on a light blue or white background with greater sensitivity for hydroxylated steroids than phosphomolybdic acid dissolved in ethanol. G.l.c. was done as described by Haslewood (1971).

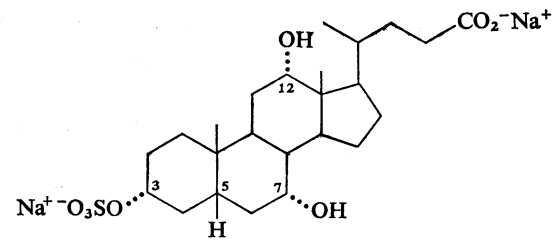
Preparation and properties of 3-sulphates of bile acids

(1) *Preparation of 3α-hydroxy acetoxy methyl esters* (*cf. Haslewood*, 1944). The fully acetylated methyl ester (1g) of cholic, chenodeoxycholic or deoxycholic acid was dissolved in methanol (20ml) containing HCl (11.8M, 1ml; AnalaR quality, Hopkin and Williams, Chadwell Heath, Essex, U.K.). The mixture was heated under reflux on a boiling-water bath for 30min, cooled, diluted with water and extracted twice with diethyl ether. The combined diethyl ether extracts were washed with water, aq. $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. The residue was a colourless gum that on t.l.c. with the solvent system benzene/acetone (25:6, v/v) showed a main spot with traces of more polar material. The product from chenodeoxycholic acid readily crystallized from light petroleum (b.p. 40–60°C)/diethyl ether (approx. 3:1, v/v) as long colourless prisms of methyl 7α-acetoxy-3α-hydroxy-5β-cholan-24-oate, m.p. 117–118°C (cf. Hauser *et al.*, 1960). The products from cholic and deoxycholic acids could not be crystallized.

(2) *Sulphation at C-3.* A 3α-hydroxy acetylated methyl ester [1g, prepared as under (1) above] was dissolved in dry diethyl ether (20ml). To another portion of dry diethyl ether (20ml) was carefully added chlorosulphonic acid (1ml) from a dry pipette. The ethereal solution of chlorosulphonic acid was at

once added to and mixed with the solution of ester and the mixture stoppered with a $CaCl_2$ desiccator tube and kept with occasional swirling at room temperature (approx. 22°C) for 30min. It was then treated with ice and water and extracted twice with diethyl ether. [Evaporation of the washed and dried combined ethereal extracts yielded gums (approx. 30–50mg) that presumably represented non-sulphated material, including fully acetylated ester that had escaped solvolysis by HCl.] The aqueous portion was carefully treated with solid Na_2CO_3 (approx. 2.5g in all) added in small portions, with mixing, until a drop of the solution gave a blue-green colour with Bromothymol Blue, used as external indicator. The solution was then evaporated to dryness on a boiling-water bath. The residue was extracted 4–5 times with ethanol/methanol and the extract was filtered. Evaporation of the filtrate left a residue that was re-extracted with ethanol. The filtered extract was evaporated to leave an almost colourless gum, presumably consisting chiefly of the sodium salt of the 3-sulphate of the otherwise fully acetylated methyl ester.

(3) *Preparation of the bile acid 3-sulphates.* The 3-sulphate acetylated methyl ester (1g) was dissolved in water (75ml) and the solution heated for about 15min on a boiling-water bath under reflux. Aq. NaOH (1M, 25ml) was then added and heating continued for a further 30min. After cooling, the solution was treated with HCl (1M) until a drop just gave a blue colour with Bromothymol Blue, used as external indicator. It was then evaporated to dryness on a boiling-water bath. The residue was extracted with ethanol/methanol 4–5 times and the extracts were filtered, combined and evaporated to dryness. The residue was extracted with ethanol, with warming if necessary, and the filtered ethanol evaporated finally *in vacuo* at about 90°C; repetition of this process appeared to remove inorganic salts. The residues in all cases were colourless gums (yield, approx. 1.1g) that could be crystallized as follows. The gum was dissolved in methanol (5ml) with water (0.25ml), and diethyl ether (15ml) was carefully added, with mixing, so as to produce an oily precipitate. On refrigeration (0–4°C) the preparation gradually formed groups of colourless needles, which could be increased by scratching, and finally became entirely crystalline; it was then collected, washed with cold methanol/diethyl ether (1:3, v/v) and dried *in vacuo* over $CaCl_2$. Material from chenodeoxycholic acid could be crystallized from ethanol. Melting points and analyses suggested that the crystalline preparations were solvated disodium salts (e.g. cholic acid 3-sulphate disodium salt, formula I). The bile acid 3-sulphates were analysed for Na^+ by flame photometry and for SO_4^{2-} after solvolysis of approx. 8mg with ethyl acetate/HCl at 37°C for at least 48h as described by Anderson *et al.* (1974).



(I)

After this solvolysis, $BaCl_2$ (0.5M, 0.2ml) was added together with approx. 0.5ml of water, and after at least 1h, $BaSO_4$ was collected on a weighed sintered-glass filter, washed with water and methanol and dried at 100°C. The organic products of solvolysis were obtained by extraction of the filtrate twice with ethyl acetate; the combined extracts were washed three times with water, dried over Na_2SO_4 and evaporated. T.l.c. with the solvent system 2,2,4-trimethylpentane/ethyl acetate/acetic acid (7:12:3, by vol.) showed that the parent acid was a minor constituent in every case. Cholic acid 3-sulphate gave at least two major spots less polar than cholic acid and deoxycholic acid 3-sulphate and chenodeoxycholic acid 3-sulphate each gave one major spot less polar than the parent acid. These products dissolved in methanol (0.4ml) and aq. NaOH (0.1M, 0.1ml) were heated on a boiling-water bath for 1h. Acidification with HCl (1.0M) gave precipitates that, after refrigeration, were collected and washed with water, yielding substances that were shown by t.l.c. and g.l.c. to consist chiefly of the parent acids.

(4) *Properties of the bile acid 3-sulphates.* (A) *Cholic acid 3-sulphate disodium salt.* This formed colourless needles when crystallized from methanol/water/diethyl ether (as above) that, after drying *in vacuo* over solid $CaCl_2$, lost form at about 220°C and cleared, with decomposition (bubbling and browning), at approx. 233°C. (Found: Na^+, 7.2; S, 4.75. Calc. for $C_{24}H_{38}O_8SNa_2,6H_2O$: Na, 7.2; S, 5.0%.) On t.l.c. with the solvent system acetic acid/3-methylbutyl acetate/water (5:2:2, by vol.) this material gave a main spot with R_F approx. 0.33 when taurocholate had R_F approx. 0.24. Traces of more polar and slightly less polar material were detectable on the chromatogram.

(B) *Chenodeoxycholic acid 3-sulphate disodium salt.* When crystallized from ethanol this gave small white prisms that, after drying *in vacuo* over solid $CaCl_2$, had m.p. 207–209°C (decomp.). (Found: Na^+ 7.3; S, 5.3. Calc. for $C_{24}H_{38}O_7SNa_2,2C_2H_5OH$: Na^+, 7.6; S, 5.3%). On t.l.c. (as above) this substance gave a single spot with R_F approx. 0.35 when taurodeoxycholate had R_F approx. 0.30.

(C) *Deoxycholic acid 3-sulphate disodium salt.* When crystallized from methanol/water/diethyl

ether, this gave colourless needles that, after drying *in vacuo* over $CaCl_2$, lost solvent at approx. 225°C and cleared, with decomposition, at 239°C. (Found: Na^+, 7.6; S, 5.1. Calc. for $C_{24}H_{38}O_7SNa_2,5H_2O$: Na^+, 7.6; S, 5.3%.) On t.l.c. (as above) this substance gave a main spot with the same R_F as the chenodeoxycholic acid sulphate and a faint spot with R_F approx. 0.23.

The above sulphates gave negative responses in the Hammarsten (HCl) test. They readily dissolved in water, aq. HCl (0.1M) or methanol; less readily in ethanol and sparingly in acetone, ethyl acetate or diethyl ether. Their infrared spectra (KBr) showed sulphate ester absorption maxima in the regions 1240–1250, 1060–1070 and 825–840 cm^{-1} (cf. Mumma, 1966); the wide bands with maxima at 1240–1250 cm^{-1} had the almost smooth outline characteristic of sulphate esters (Anderson *et al.*, 1974). On small columns of Sephadex LH-20 (Haslewood & Haslewood, 1972) approximate volumes of chloroform/methanol (1:1, v/v) that eluted most of the material were: cholic acid 3-sulphate, 7–12ml; chenodeoxycholic acid 3-sulphate, 4–9ml; deoxycholic acid 3-sulphate, 8–13ml.

(5) *Solvolysis.* Solvolysis, as described above, was carried out on cholic acid 3-sulphate with butanone instead of ethyl acetate, resulting in a similar formation of a number of products mainly hydrolysable to cholic acid. Some of the methods described in the careful study by Goren & Kochansky (1973) were also tried; for example, cholic acid 3-sulphate disodium salt (10.2mg) and dioxan [0.5ml, purified as described by Goren & Kochansky (1973)] were treated with a saturated aq. solution of hydrazine dihydrochloride (50μl). After 2h, with occasional mixing, at room temperature (approx. 22°C), dioxan was removed in a stream of N_2 and the residue treated with water (1–2ml). Water was decanted and found to contain SO_4^{2-} corresponding to about 50% of complete solvolysis. The organic (water-insoluble) product (7.0mg) was found by t.l.c. to contain at least three substances; cholic acid was a minor constituent. This mixture did not yield cholic acid as the chief product on alkaline hydrolysis.

Discussion

The work described above shows that specifically esterified bile acid sulphate esters can be made and that protecting acyl groups can be removed by alkaline hydrolysis without affecting the sulphate esters. There seems no reason to think that conjugated glycine or taurine will be removed in the conditions of hydrolysis used. Solvolysis of the 3-sulphate ester groups, leading to the parent acids, has not so far been effectively done in a one-step process; however, ethyl acetate/HCl solvolysis followed by hydrolysis does seem to offer a suitable procedure. We cannot explain the formation of esters or other artifacts on solvolysis. In our considerable experience with ethyl acetate/HCl solvolysis of side-chain sulphate esters (Anderson *et al.*, 1974) we have found acetylation, but only to a minor extent; acetylation will not, of course, explain the formation of artifacts when solvolysis is carried out with butanone or dioxan. During early attempts at isolation of the 3-sulphates, we made the mixture, after sulphation, ammoniacal and then evaporated the product to dryness, leaving a residue nominally comprising a mixture of $(NH_4)_2SO_4$, NH_4Cl and the NH_4^+ salts of the 3-sulphate esters. Extraction of this residue with acetone or even ethanol resulted, on evaporation of the filtered extract, in almost complete solvolysis of the 3-sulphates with precipitation of $(NH_4)_2SO_4$. The organic material formed was mainly the original 3-hydroxy acetoxy methyl ester. The conditions of solvolysis here resemble those described by Goren & Kochansky (1973) and the fact that artifacts were not formed suggests that a free $-CO_2H$ group may be important in their generation during the solvolysis of the bile acid 3-sulphates.

Stiehl *et al.* (1975) describe the sulphation of cholic and chenodeoxycholic acids. They give no analyses or details of the chemistry of their products and it is difficult therefore to judge the biological relevance of their findings. Makino *et al.* (1975) likewise describe the presence of bile acid sulphates in bile, blood and urine of patients with hepatobiliary disease, also without reference to chemically defined compounds. It seems to us that a foundation of chemistry is needed for the further investigation of sulphated bile salts in physiological or clinical studies.

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