Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.
Brunngraber, E. G. & Abood, L. G. (1960). *J. biol. Chem.* **235**, 1847.
Cremer, J. E. (1960). *Biochim. biophys. Acta*, **41**, 155.
Geiger, A. (1940). *Biochem. J.* **34**, 465.
Hochstein, P. & Cohen, G. (1960). *J. Neurochem.* **5**, 370.
Johnson, M. K. (1960). *Biochem. J.* **77**, 610.
Johnson, M. K. (1961). *Biochem. J.* **79**, 24P.
Krimsky, I. & Racker, E. (1949). *J. biol. Chem.* **179**, 903.
Krimsky, I. & Racker, E. (1954). *Fed. Proc.* **13**, 245.
Muntz, J. A. & Hurwitz, J. (1951). *Arch. Biochem. Biophys.* **32**, 124.
Rapkine, L. (1938). *Biochem. J.* **32**, 1729.
Roodyn, D. B. (1956). *Biochem. J.* **64**, 368.
Rossiter, R. J. (1957). *Canad. J. Biochem. Physiol.* **35**, 579.

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Comparative Studies of 'Bile Salts'

14. ISOLATION FROM SHARK BILE AND PARTIAL SYNTHESIS OF SCYMNOL*

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The name 'scymnol' was given by Hammarsten (1898) to an alcohol believed by him to occur as a sulphate in the bile of the shark *Scymnus borealis* (*Somniosus microcephalus* Schn.). 'Scymnol' has been isolated after hydrolysis with alkali of the bile salts of about ten species of selachians. It has been shown (Fieser & Fieser, 1959; Cross, 1960) that this substance has the formula (III*a*). Haslewood (1951) suggested that the compound now recognized as (III*a*) might be an artifact, the oxide ring arising by elimination of SO_4^{2-} ion between an –OH group and $-O\cdot SO_3^-$ during alkaline hydrolysis. This suggestion was shown (Briggs & Haslewood, 1961) to be correct, for direct oxidation of the sulphate from shark bile gave dehydrocholic acid (IV*b*); this work is here described in full. It follows that the native bile salt (III*b*) is the sulphate of a hexahydric alcohol which, in agreement with Dr A. D. Cross, we call 'scymnol' (III*c*), the substance (III*a*) being renamed 'anhydroscymnol'.

Scymnol, $C_{27}H_{48}O_6$, has now been isolated from a sample of shark bile salt sulphate and has also been made by partial synthesis from cholic acid.

RESULTS

The shark bile sulphate used was a sample, the gift of Professor S. Bergström, from Professor O. Hammarsten's collection: it was labelled 'scymnol-sulphuric acid sodium salt', and showed a single spot on paper chromatograms.

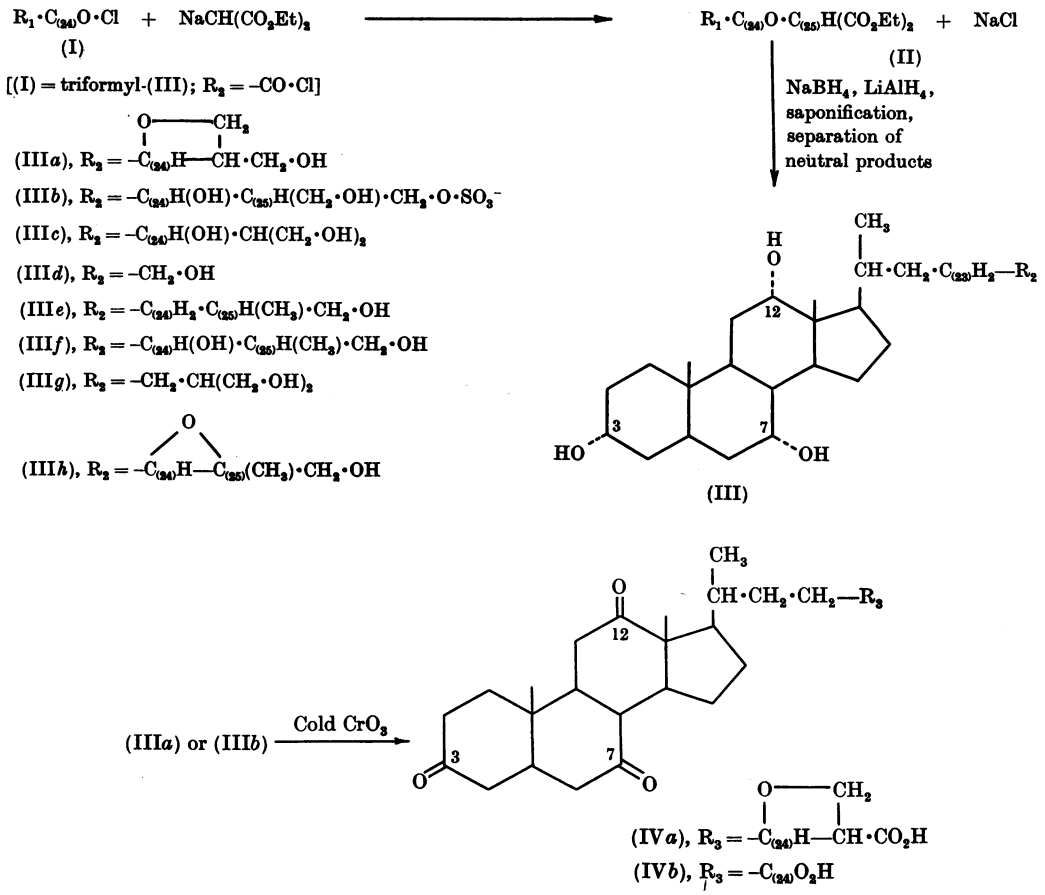
Cold chromic acid oxidation of this material gave dehydrocholic acid (IV*b*) in a yield of about 26%. When anhydroscymnol (III*a*) was treated in the same way, the product (yield, about 40%) was the known 'scymnol acid' (anhydroscymnol acid, $C_{27}H_{38}O_6$), in which the oxide ring is retained (Bergmann & Pace, 1943) and which must have the structure (IV*a*).

For removal of the sulphate ester group, scymnol sulphate was first partially acetylated and the crude product was treated with dry dioxan–trichloroacetic acid (Cohen & Oneson, 1953). The water-insoluble material obtained was hydrolysed with alkali and purified by partition chromatography. The product, crystallized from moist solvents, was much more 'polar' on paper chromatograms than anhydroscymnol: elementary analysis suggested that it was a dihydrate, $C_{27}H_{48}O_6,2H_2O$, of *scymnol* (III*c*).

In the partial synthesis, triformylcholoyl chloride (I) was condensed with diethyl sodiomalonate. The partially purified product, containing (II), was reduced, first with $NaBH_4$ in dimethylformamide and then with $LiAlH_4$ in tetrahydrofuran. Saponification followed; the neutral fraction was separated and four crystalline substances and a gum were obtained from it, as described below.

A principal product was 3α,7α,12α,24-tetrahydroxycholane (III*d*), which could easily be isolated by direct crystallization. Paper chromatography showed that at least four other compounds were present, and three of these were isolated after chromatography on alumina. A sample obtained by this process, and showing a single spot on paper chromatograms corresponding to one given by the scymnol described above, was finally purified by partition chromatography. Crystalline material,

* Part 13: Haslewood (1961).



identical with scymnol (III*c*) in its behaviour and infrared absorption (Fig. 1*A*), was finally obtained in a yield (calculated on cholic acid as starting material) of about 0·5%.

A third crystalline substance from the partial synthesis ran at the same rate on paper chromatograms as a 3α,7α,12α,27-tetrahydroxycoprostane (III*e*), made by $LiAlH_4$ reduction of the ethyl 3α,7α,12α-trihydroxycoprostanate (mainly 25α-) from alligator bile (Haslewood, 1952; Bridgwater, 1956). It also gave (approximately) the expected analysis, for $C_{27}H_{48}O_4$. Although its infrared spectrum (Fig. 1*B*) was almost identical with that of the tetrahydroxycoprostane, both were so similar to that (Fig. 1*C*) of the compound $C_{27}H_{48}O_5$ described below as to throw doubt on this criterion of identity, in the present case. The substance $C_{27}H_{48}O_4$ may of course differ from the alcohol from ethyl 3α,7α,12α-trihydroxycoprostanate in its configuration at C-25: the latter alcohol appeared, from its melting point, to be a mixture of epimers, as would be expected from the properties of the original acid (Bridgwater, 1956).

The remaining partially synthetic material showed mobility on paper close to that of 26-deoxyscymnol [(III*f*), 'dihydroscymnol'; Cross, 1960] and prolonged chromatography revealed that it was a mixture. A crystalline substance was isolated from this: analysis supported the formula $C_{27}H_{48}O_5$, with some degree of hydration. This compound, after prolonged chromatography on paper, showed a mobility slightly different from that of 26-deoxyscymnol: it may be epimeric (at C-24 or C-25 or both) with this substance, and it is also possible that it is 24-deoxyscymnol (III*g*).

The final gummy material from the partial synthesis was not separated further into its component substances: it gave a remarkably intense response in the Hammarsten (HCl) reaction.

EXPERIMENTAL

General. Al_2O_3 and 20% CrO_3 were as described by Bridgwater (1956). Melting points (Kofler block) are uncorrected. Microanalyses were by Weiler and Strauss, Oxford; as is usual in compounds of the type described here,

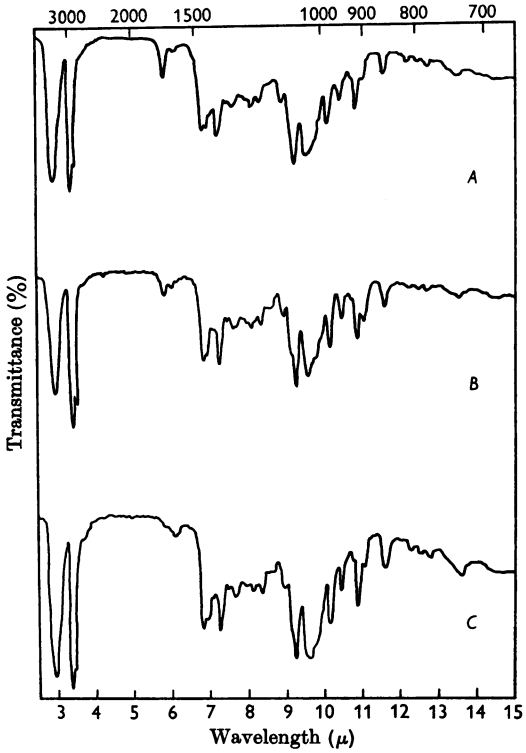


Fig. 1. Infrared spectra in potassium bromide: *A*, Scymnol (3α,7α,12α,24ξ,26,27-hexahydroxycoprostane); *B*, (probably) 3α,7α,12α,24ξ-tetrahydroxycoprostane; *C*, (probably) 26-deoxyscymnol (3α,7α,12α,24ξ,26-pentahydroxycoprostane). (Transmittance at 4000 cm.$^{-1}$ in all cases was above 96%.) A small 'carbonyl' band (at about 5·84 μ) was found in all samples of scymnol examined; it may be due to impurity.

some hydration was evident from the analytical figures in every case, although crystals were dried to constant wt. *in vacuo* at 80–100° before analysis. Infrared spectroscopy was as by Haslewood (1961), with methanol or acetone as preliminary solvents.

Paper chromatography. For examination of the products of partial synthesis (at 40°), Whatman 3MM paper (previously washed with methanol) was used, with the system (parts by vol.): di-*n*-butyl ether (The Distillers Co. Ltd., redistilled, 80)–'amyl acetate' (The Distillers Co. Ltd. 'confectionary grade', redistilled, b.p. 141–142°, 20)–acetic acid (70)–water (30). When this solvent mixture had been equilibrated, the paper was washed with the mobile (dibutyl ether–amyl acetate) phase, briefly dried at room temp., 'spotted' with the substances to be run and hung in an atmosphere of both phases for 90 min. at 40°. 'Chromatography' was ascending and 'prolonged chromatography' descending. Approximate representative values of R_F were: scymnol, 0·27; 26-deoxyscymnol, 0·57; anhydrosymnol, 0·49; 3α,7α,12α,24-tetrahydroxycholane, 0·67; 3α,7α,12α,27-tetrahydroxycoprostane, 0·83.

Partition chromatography ('reversed phase') was done in general as described by Haslewood (1961). The column was made up with Hostalen W [Farbwerke Hoeschst A.-G., Frankfurt (M), Germany] which had been extracted in a Soxhlet apparatus for 8 hr. with methanol and then dried at about 70°. 2-Ethylhexan-1-ol (redistilled, b.p. 183–184°)–$CHCl_3$ (1:1, v/v) was the stationary and 46% (v/v) methanol–water the moving phase (Norman, 1953).

Isolation of scymnol from its sulphate

The sulphate (50 mg.) was dissolved in acetic acid (0·1 ml.) and refluxed for 45 min. with acetic anhydride (0·5 ml.). Addition of methanol and thorough evaporation (finally *in vacuo* over NaOH) left a residue (67 mg.) of partially acetylated scymnol sulphate. To this was added 0·25 ml. of a solution (40%, w/v) of trichloroacetic acid (redistilled) in dry dioxan. When solution was complete, further dioxan (0·75 ml.) was added. The viscous solution set to a gel, then liquefied during 1 hr. After several days at room temp. dioxan was removed at 50–55° under N_2, water was added and the mixture was extracted three times with ethyl acetate (about 3 × 3 ml.). The combined extract was washed with water and evaporated, leaving a gum (158 mg.) containing trichloroacetic acid and partially acetylated scymnol. The combined aqueous phase gave, with excess of aqueous 2M-$BaCl_2$, 17·0 mg. of $BaSO_4$ (yield, 83% of theoretical). The acetylated scymnol was heated under reflux with approx. N-KOH in ethanol (sufficient to neutralize the trichloroacetic acid and, in addition, 2 ml.) for 2 hr. The product was extracted with butan-1-ol–ethyl acetate (1:1); evaporation of the extract left crude scymnol (48 mg.) as a gum. Purification was effected by reversed-phase partition chromatography on Hostalen (9 g.) with 2-ethylhexan-1-ol–chloroform (6 ml.) and 46% (v/v) methanol–water as described above. The portion (18–30 ml.) of effluent which contained scymnol was evaporated and the residue crystallized twice from methanol–ethyl acetate. Crystallization required the presence of water in the solvent mixture. *Scymnol dihydrate* formed fine white needles of m.p. about 120–123°; sometimes crystals re-formed and these had m.p. about 190°. $[\alpha]_D^{25}$ + 34 ± 2° (*c* 0·9 in ethanol), Hammarsten test, purple (Found: C, 67·6; H, 10·15, Analysis calc. to wt. of undried sample: C, 64·1; H, 9·9. $C_{27}H_{48}O_6$ requires C, 69·2; H, 10·3; $C_{27}H_{48}O_6,2H_2O$ requires C, 64·3; H, 10·3%). The yield of crystalline scymnol dihydrate was 36%, calculated on the basis of the original scymnol sodium sulphate, or 43% on the basis of $BaSO_4$ found.

Dehydrocholic acid from scymnol sulphate

Sodium scymnol sulphate (50 mg.) was dissolved in acetic acid (0·5 ml.) and 20% CrO_3 (0·35 ml.) was added at 0°. After 3 hr. at 25° with occasional mixing, water and NaCl (excess) were added and the mixture kept at about 5° overnight. The precipitate was collected, washed with water and partitioned between water and ethyl acetate. Further extraction with ethyl acetate and evaporation of the extracts left dehydrochloric acid, which crystallized as long needles (9 mg., m.p. 209–217°) from aqueous ethanol. Yield, 26%. Repeated crystallization raised the m.p. to 224–231° (decomp.). The infrared spectrum was identical with that of authentic dehydrocholic acid.

Anhydroscymnol (50 mg.), simultaneously subjected to the procedure described above, gave anhydroscymnol acid

(21 mg., m.p. 221–224°, from aqueous ethanol). Yield, 40%. Recrystallization from ethanol–toluene raised the m.p. to 228–231°. The infrared spectrum differed from that of dehydrocholic acid and showed the strong absorption at about 961 cm.$^{-1}$ attributed to the oxide ring (Cross, 1960).

Partial synthesis of scymnol and of substances probably (III*d*), (III*e*) *and* (III*f*)

A suspension of diethyl sodiomalonate was prepared from a finely divided suspension of sodium (14 g.) in boiling toluene (400 ml.) and diethyl malonate (108 ml.). Triformylcholoyl chloride (46 g.) in toluene (150 ml.) was added at room temp. and the mixture warmed on a water bath (about 60°) for 1 hr. and left overnight. The mixture was poured into water and ether in a separating funnel; the organic phase was washed with 2N-HCl (twice), water (once), aqueous $NaHCO_3$ (three times) and water (three times). Evaporation of the ether and toluene left a gum, which was dissolved in dimethylformamide (150 ml.). The solution, cooled in ice–water, was treated with $NaBH_4$ (8 g.), added during 0·5 hr. After 2 hr. at room temperature, the mixture was poured into water (about 200 ml.) and extracted with ethyl acetate, which was washed to neutrality and dried (Na_2SO_4). The product (48 g., oil) left on removal of the ethyl acetate was dissolved in dry tetrahydrofuran (450 ml.) and treated during 2 hr. with $LiAlH_4$ (20 g.); it was then left for 2 days. The mixture was poured into water (2 l.), acidified (10N-HCl) and extracted with ethyl acetate (×2). The ethyl acetate extract was washed with $NaHCO_3$ (satd. aqueous) and evaporated. The residual oil was dissolved in methanol (approx. 200 ml.) and treated with 3N-NaOH (30 ml.) on a steam bath for 0·5 hr. The product was poured into water and extracted with ethyl acetate; the extract was washed with aqueous $NaHCO_3$ and water (×3), giving a solution which, on evaporation to about 100 ml. and cooling, yielded crystals (about 9·5 g.). These had m.p. 226–227°, not depressed by authentic 3α,7α,12α,24-tetrahydroxycholane, and ran at the same rate as this substance on paper chromatograms. The filtrate (approx. 100 ml. of ethyl acetate) was mixed with acetone (100 ml.) and the mixture run on a column of Al_2O_3 (700 g.) made up in acetone. Acetone (3 l.) was run through the column, followed by acetone containing 10% (v/v) of methanol (3 l.). These two eluates were combined and evaporated to give *product A* (4 g.). Acetone with 20% (v/v) methanol (4 l.) and 50% (v/v) methanol (2 l.) gave fractions which paper chromatography showed to contain 3α,7α,12α,24-tetrahydroxycholane as the chief constituent. Methanol (4 l.) eluated most of the material remaining on the column. Evaporation of the methanol left a product which was dissolved in acetone containing 20% (v/v) methanol and introduced on a column of Al_2O_3 (150 g.) in the same solvent mixture. Methanol–acetone (1 l.; 20%, v/v) was run through the column, followed by the same solvent mixture (500 ml.); this second eluate on evaporation yielded a resin (0·24 g.; *product B*). Methanol–acetone (1 l., 30%, v/v) was passed through the column followed by methanol–acetone (1 l.; 40%, v/v), methanol–acetone (2 l.; 60%, v/v) and finally methanol (1 l.). Evaporation of the last combined eluates (5 l.) gave a resin (0·84 g.) which, as paper chromatography showed, consisted largely of scymnol. For final purification, this resin (419 mg.) was separated as described above on Hostalen (72 g.) with 2-ethylhexan-1-ol–$CHCl_3$ (48 ml.) and methanol–water. Scymnol (166 mg.) was eluted (as a single peak in the graph wt. eluted/ml. of eluate) by 180–295 ml. of moving phase. Crystallization from methanol–ethyl acetate (containing water) gave fine white needles of the same m.p., appearance and infrared spectrum as the material from shark bile; the mixed m.p. with this substance showed no depression. $[\alpha]_D^{25} +32 \pm 1°$ (*c* 0·9 in ethanol). Yield, first (purest) crop, 74 mg. A second crop (80 mg.) crystallized from the liquors.

Product A. On rechromatography (140 g. of Al_2O_3; column made in ether) this gave on elution with ether (about 600 ml.) a fraction which, on evaporation, left a residue which crystallized from ethyl acetate as needles (about 30 mg.) of m.p. 177–181°. The mixed m.p. with a (neutral) substance of m.p. 178–183° [probably 3α,7α,12α,-27-tetrahydroxycoprostane (III*e*)], made by $LiAlH_4$ reduction of ethyl 3α,7α,12α-trihydroxycoprostanate from alligator bile, was 173–178° (Found: C, 73·6; H, 10·6. $C_{27}H_{48}O_4$ requires C, 74·5, H, 11·1%: wt. was lost on drying at 100° *in vacuo*). Further elution with methanol–acetone gave fractions containing some 3α,7α,12α,24-tetrahydroxycholane.

Product B. This gave in the Hammarsten test an immediate red colour, followed by a deep-purple mixture containing a dark precipitate. Product B, on recrystallization from ethyl acetate–methanol, ethyl acetate–acetone and finally acetone gave crystals (about 12 mg.) of m.p. 171–173°, which gave a typical (purple) Hammarsten test. The mixed m.p. with 26-deoxyscymnol (III*f*, m.p. 179–182°) was 168–172° (Found: C, 70·7; H, 10·6. $C_{27}H_{48}O_5$ requires C, 71·7; H, 10·6%). The mother liquors on paper chromatography were shown to contain a mixture of at least three compounds, all more 'polar' than 3α,7α,12α,24-tetrahydroxycholane. The final gum showed the response in the Hammarsten test given by product B.

DISCUSSION

Chemical. The work described, together with that of Cross (1960, 1961), leaves no doubt that scymnol is (III*c*); the only undetermined feature is the configuration at C-24. Both C-24 epimers may, of course, occur in biles. We think that our oxidation experiment (III*b* → IV*b*) provides satisfactory evidence that the sulphate group in the bile salt is in fact at C-27 (as III*b*) and not at C-24.

The partial synthesis succeeded by the use of the newer metal hydrides in exploiting a substance of type (II), hitherto regarded by us as intractable for adding to chain length. The yield of neutral substances having the extra carbon atoms was very poor, the chief product being tetrahydroxycholane (III*d*). We are not sure whether this was due to incomplete condensation [(III*d*) arising by reduction of (I)], or whether the conditions of reduction were such as to cause splitting at C-24–C-25 in (II). In earlier experiments, we found that boiling the condensation product with $NaBH_4$ in methanol gave a high yield of tetrahydroxycholane and little scymnol. Direct reduction of (II) with $LiAlH_4$ also gave this result.

The C_{27} products of the partial synthesis were, with the exception of scymnol itself, not characterized to our full satisfaction. Such characterization must involve the separation of optical isomers at C-24 or C-25 or both, and this task was outside the scope of the present work. Identification, such as it was, depended chiefly on melting points, analyses and paper chromatography. Wootton (1953) has pointed out that the infrared spectra of substances with the cholic acid nucleus (as in III) may be very similar, and the present work shows that some spectra (e.g. Fig. 1*B*, *C*) from quite different substances of this type are nearly identical.

Biological. Apart from the suggestion of Fieser & Fieser (1949, 1959) and until the publication of Cross (1960), anhydroscymnol was thought to have the formula (III*h*). This structure was in agreement with the idea that bile-salt evolution had taken a course which led from bile alcohols of types such as (III*e*) to the hydroxycoprostanic acids [e.g. (III), $R_2 = -C_{(24)}H_2 \cdot CH(CH_3) \cdot CO_2H$] and thence, by β-oxidation at C-24, to the 'modern' C_{24} bile acids. The structure of scymnol now elucidated may imply that the actual course of events was more complex. Perhaps there have been at least two routes to 'modern' bile acids, one as set out above and the other proceeding by direct oxidation at C-24 of substances like scymnol, or possibly the derived carboxylic acids [e.g. (III), $R_2 = -C_{(24)}(OH) \cdot CH(CH_2 \cdot OH) \cdot CO_2H$], to the C_{24} acids. This second route might not include the known hydroxycoprostanic acids, still in use as bile salts, but of course might have involved bile alcohols of types (III*e*) or (III*f*). The general notion that there might have been more than one evolutionary pathway between cholesterol and cholic acid is entirely in accord with what seems obvious at present: that cholic acid has been produced by evolution at least twice (Haslewood, 1959). It is an aim of the present studies to attempt to elucidate such evolutionary pathways, so far as they can be regarded as still represented by substances in use as bile salts of existing species.

It has already been established that both the 3α,7α,12α-trihydroxycoprostanic acids, epimeric at C-25, are readily converted by the rat into cholic acid (Bridgwater & Lindstedt, 1957). The same reaction occurs with rat-liver homogenates (Bergström, Bridgwater & Gloor, 1957) and mitochondria (Briggs, Whitehouse & Staple, 1961), and such homogenates from rat and mouse liver can make 3α,7α,12α,27-tetrahydroxycoprostane (III*e*) from 3α,7α,12α-trihydroxycoprostane [(III), $R_2 = -CH_2 \cdot CH(CH_3)_2$]. Mouse-liver homogenates convert the above tri- and tetra-hydroxycoprostanes into 3α,7α,12α-trihydroxycoprostanic acid (Danielsson, 1960). The alligator converts cholesterol into this acid (Briggs, Whitehouse & Staple, 1959), which (as a taurine conjugate) is in this species a major bile salt. It is difficult to believe that all these reactions, established by direct experiment as occurring *in vivo*, are unrelated to pathways of bile-salt evolution.

SUMMARY

1. A specimen of 'scymnol sulphuric acid, sodium salt', from the original collection of Prof. O. Hammarsten, has been oxidized with cold chromic acid to yield dehydrocholic acid [3,7,12-trioxocholanic acid (IV*b*)]. The same conditions applied to anhydroscymnol [(III*a*), formerly called 'scymnol')] gave the corresponding (C_{27}) anhydroscymnol acid (IV*a*). Hence, scymnol sulphate has the structure (III*b*).

2. Dioxan–trichloroacetic acid treatment of partially acetylated scymnol sulphate gave, after complete hydrolysis and purification, a hexahydric alcohol, i.e. a dihydrate, $C_{27}H_{48}O_6,2H_2O$, of the native bile alcohol, *scymnol* [(III*c*), 3α,7α,12α,24ξ,-26,27-hexahydroxycoprostane]. This is the characteristic bile alcohol of those selachians (sharks and rays) from which anhydroscymnol has been isolated after alkaline hydrolysis, anhydroscymnol being an artifact of the alkali treatment.

3. Condensation of triformylcholoyl chloride with diethyl sodiomalonate gave a product which was reduced by sodium tetrahydroborate in dimethylformamide and then by lithium tetrahydroaluminate in tetrahydrofuran. From the neutral substances isolated after saponification we obtained scymnol, 3α,7α,12α,24-tetrahydroxycholane (III*d*) and two other crystalline substances which may be 3α,7α,12α,27-tetrahydroxycoprostane (III*e*) and 26-deoxyscymnol (III*f*), or epimers at C-24 or C-25 of these compounds.

4. Scymnol thus becomes the first bile alcohol of virtually known structure. Consideration has therefore been given to possible pathways of evolution of bile salts, which have led to bile alcohols with side chains ending in $-C_{(24)}H(OH) \cdot CH(CH_2 \cdot OH)_2$, coprostanic acids whose side chains end as $-C_{(24)}H_2 \cdot CH(CH_3) \cdot CO_2H$ and finally to modern C_{24} bile acids, side-chain $-C_{(24)}O_2H$.

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REFERENCES

Bergmann, W. & Pace, W. T. (1943). *J. Amer. chem. Soc.* **65**, 477.
Bergström, S., Bridgwater, R. J. & Gloor, U. (1957). *Acta chem. scand.* **11**, 836.
Bridgwater, R. J. (1956). *Biochem. J.* **64**, 593.
Bridgwater, R. J. & Lindstedt, S. (1957). *Acta chem. scand.* **11**, 409.
Briggs, T. & Haslewood, G. A. D. (1961). *Biochem. J.* **79**, 5P.
Briggs, T., Whitehouse, M. W. & Staple, E. (1959). *Arch. Biochem. Biophys.* **85**, 275.
Briggs, T., Whitehouse, M. W. & Staple, E. (1961). *J. biol. Chem.* **236**, 688.
Cohen, S. L. & Oneson, I. B. (1953). *J. biol. Chem.* **204**, 245.
Cross, A. D. (1960). *Proc. chem. Soc., Lond.*, p. 344.
Cross, A. D. (1961). *J. chem. Soc.* p. 2817.
Danielsson, H. (1960). *Acta chem. scand.* **14**, 348.
Fieser, L. F. & Fieser, M. (1949). *Natural Products Related to Phenanthrene*, 3rd ed. p. 113. New York: Reinhold Publishing Corp.
Fieser, L. F. & Fieser, M. (1959). *Steroids*, pp. 431–432. New York: Reinhold Publishing Corp.
Hammarsten, O. (1898). *Hoppe-Seyl. Z.* **24**, 322.
Haslewood, G. A. D. (1951). *Symp. biochem. Soc.* **6**, 83.
Haslewood, G. A. D. (1952). *Biochem. J.* **52**, 583.
Haslewood, G. A. D. (1959). *Ciba Foundation Symp., Biosynthesis of Terpenes and Sterols*, p. 207. London: J. and A. Churchill Ltd.
Haslewood, G. A. D. (1961). *Biochem. J.* **78**, 352.
Norman, A. (1953). *Acta chem. scand.* **7**, 1413.
Wootton, I. D. P. (1953). *Nature, Lond.*, **172**, 954.

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The Interconversion of Amino Acids after their Incorporation into Haemoglobin and Myosin

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Kruh, Dreyfus, Schapira & Padieu (1957) have shown that haemoglobin is not uniformly labelled when either radioactive glycine or phenylalanine is injected (Kruh, Dreyfus & Schapira, 1960), but that the labelling becomes uniform when the red blood cells become older. These results have suggested several hypotheses; one of them was that haemoglobin does not remain inert and undergoes intramolecular changes during the life of the red cells.

It has been observed that myosin is sufficiently stable to reflect the life span of the myofibrils (Dreyfus, Kruh & Schapira, 1960). Comparison of the specific activities of glycine and serine, after the injection of radioactive glycine, and of the specific activities of phenylalanine and tyrosine, after the injection of radioactive phenylalanine, has suggested that a conversion of glycine into serine and of phenylalanine into tyrosine could have occurred after the incorporation of the precursor amino acids, during the life of the red blood cells and of the myofibrils (a preliminary report of these observations has been published; Schapira, Dreyfus & Kruh, 1958). Similar conversion has been shown to occur in collagen (Stetten, 1949; Piez & Likins, 1957; Van Slyke & Sinex, 1958; Sinex, Van Slyke & Christman, 1959; Robertson, Hiwett & Herman, 1959; Green & Lowther, 1959; Chvapil & Cmuchalova, 1960). Our study is favourably influenced by the fact that haemoglobin and myosin are not renewed or very slowly renewed, which makes it possible to exclude rapid exchange between bound and free amino acids.

Preliminary experiments have shown that when the animals were fed a normal diet the ratios of the specific activities of glycine to serine in myosin rapidly became unity after the injection of radioactive glycine. Besides, Neuberger (1951), using rats and rabbits on a normal diet, found a ratio between specific activities of protoporphyrin and globin glycine constant with time. It is likely that the equilibration had already occurred after 2 days. On the other hand, these ratios approached unity much more gradually when the rats were fed a low-protein diet; the interconversion of amino acids after their incorporation in protein is thus more readily followed.

METHODS

Animals. The experiments were carried out with white rats of Wistar strain, weighing between 200 and 300 g. They were given a diet poor in protein, i.e. containing 8% of a mixture of animal and vegetable protein. The rats showed neither loss of weight nor anaemia. An estimation of the haemoglobin was made in each experiment. Twenty-four rats were injected intraperitoneally with [2-^{14}C]glycine and [3-^{14}C]phenylalanine at a dosage of 4 μc