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The Preparation of Chenodeoxycholic Acid and Its Glycine and Taurine Conjugates

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The preparation of chenodeoxycholic acid is described in detail. 3α,7α-dihydroxy-12-keto-5β-cholanic acid is prepared from cholic acid and reduced by the Huang-Minlon modification of the Wolff-Kishner procedure. The crude acid is methylated and chromatographed on alumina using ethyl acetate-benzene mixtures as eluants. Small fractions are collected and examined by thin layer chromatography. Those fractions containing the pure methyl ester are pooled and saponified. The chromatographically pure acid crystallizes easily from ethyl acetate-heptane, m.p. 119°. The net yield is 10–15 %. The intermediates in the synthesis have been purified chromatographically; melting points and thin layer chromatographic behaviour are given.

Methods for preparing sodium taurochenodeoxycholate and sodium glycochenodeoxycholate in a high state of purity are presented.

Chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanic acid, one of the dominant bile acids in human bile [1], was isolated over 30 years ago by Windaus *et al.* [2] and Wieland and Reverey [3]. Chenodeoxycholic and deoxycholic acids occur together in most animal bile, and because of the apparent impossibility of separating these dihydroxy acids chromatographically, most workers have prepared chenodeoxycholic acid by chemical conversion of cholic acid. Fieser and Rajagopolan [4,5] described an excellent method for the preparation of 3α,7α-dicetoxy-12-keto-5β-methyl cholanate from cholic acid and this compound can be reduced by the Wolff-Kishner reaction to give chenodeoxycholic acid in fair yield, as described by these workers. Anderson *et al.*[6] subsequently used the Huang-Minlon modification of the Wolff-Kishner reaction to prepare chenodeoxycholic acid in a similar fashion.

Although this method has been used by many workers, the reaction was recently studied in great detail by Hauser, Baumgartner and Meyer [7] because of difficulties in obtaining a crystalline product. Indeed, both Fieser and Rajagopolan [4] and Anderson *et al.*[6] had commented on the unusual manner in which chenodeoxycholic "crystallized" from ethyl acetate, the solvent gener-

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ally used for this purpose. Hauser *et al.* [7] pointed out the difficulty of acetylating the 7α-hydroxyl group as well as the resistance of the 7α-acetoxy group to hydrolysis. By careful chromatographic separation of intermediates, however, they were able to prepare chenodeoxycholic acid in a high state of purity, with a m.p. of 145—148°. An alternate method for the preparation of chenodeoxycholic acid by desulfuration of the 12 thioketal-derivative was published recently by Sato and Ikekawa [8].

For studies concerning the detergent properties of bile salts [9], large quantities of chenodeoxycholic acid were needed and we have therefore studied its preparation in some detail. The development of solvent systems for thin layer silicic acid chromatography which combined high resolution with rapid development [10,11], enabled us to check our reaction products conveniently.

This report describes a method for the preparation of chenodeoxycholic acid in fair yield (10—15 % with respect to cholic acid) from 3α,7α-dihydroxy-12-keto-5β-cholanic acid, which is relatively simple, quite reproducible in our hands, and which gives a product which by usual criteria is pure. This report would not be justified but for the observation that several of the reported methods yield an impure product and for the discovery of a hitherto undescribed crystalline form, m.p. 119°, which is obtained when the chromatographically pure product is crystallized from ethyl acetate-heptane. The steps in the preparation are shown in Fig. 1.

Preparation of the glycine and taurine conjugates of chenodeoxycholic acid by a modification of the method of Norman [12] is described. A chromatographically pure taurochenodeoxycholate is prepared by a combination of ion exchange procedures and liquid-liquid extraction procedures. Liquid-liquid extraction procedures are used for the purification of glycochenodeoxycholate but the final preparation is about 95 % pure. However, a simple adsorption chromatographic procedure is described for isolation of chromatographically pure glycochenodeoxycholate.

MATERIAL AND METHODS

The following solvents were used: di-ethyl ether (ether), dried over KOH and distilled; benzene, dried over KOH and distilled at 79—80°; dioxane (*puriss.*, E. Merck AG, Darmstadt, Germany) used without purification but kept over 4A molecular sieves (Linde Air Products Co., New York); methanol, dried over K_2CO_3 and distilled at 65°; heptane (May & Baker, Ltd., Dagenham, England) used without purification; pyridine, dried over KOH and distilled at 115°; acetic anhydride (Riedel de Haën, Hannover Germany) used without purification; tri-butyl amine (Merck) distilled at 209—215°; ethyl chlorocarbonate (Merck) distilled at 91—92°; and ethyl acetate, dried over $CaCl_2$ and K_2CO_3 and distilled. Methanol for esterifications was dried for several hours with 4A molecular sieves, then filtered.

Alumina (aluminum oxide for chromatography, WOELM, neutral, activity level I, M. Woelm, Eschwege, Germany) was inactivated to the desired activity level (Brockmann) by equilibrating with water according to the manufacturer's directions. Silicic acid (Mallinkrodt Chemical Works, New York) was activated at 120° for 24 h.

Dowex 50-W, X2 (Dow Chemical Co., Midland, Michigan) was cycled repeatedly with 1 N NaOH and 1 N HCl until the washings were colorless. Melting points, were determined with a calibrated aluminium block apparatus and are uncorrected. Before m.p. determinations, solids with a m.p. over 100° were dried for 24 h *in vacuo* in a drying pistol at 100° using phosphorus pentoxide as desiccant. Those with m.p. under 100° were dried for 24 h *in vacuo* at room temperature over phosphorus pentoxide.

Thin-layer chromatography (TLC) according to Stahl [13] was performed on layers of Silica Gel G (Merck) prepared with a commercial apparatus (C. Desaga GmbH., Heidelberg, Germany) as described [10,11].

For the numerous evaporation steps which were performed in the preparation of the conjugates and which had to be carried out without warming the reaction mixture to above about 37°, a rotary evaporator with cooling condensor and adjustable air leak (manufactured by W. Büchi, Flawil, Switzerland) proved to be of great value.

EXPERIMENTAL

The Roman numerals refer to Fig. 1.

Cholic acid, I, (3*α*,7*α*,12*α*-trihydroxy-5*β*-cholanic acid) was purchased from Riedel de Haën. As the acid was about 95 % pure by TLC, containing 2 % deoxycholic and 2 % chenodeoxycholic acid, it was not further purified. The acid can, however, be purified from its dihydroxy-contaminants by crystallization. 100 g cholic acid is refluxed in 1200 ml absolute ethanol until dissolved, and 2000 ml petroleum ether is added. After standing two days at room temperature, the solution is filtered to collect the fine crystalline precipitate. These crystals are about 99 % pure, containing less than 1 % of dihydroxy-components.

Methyl cholate, II (3*α*,7*α*,12*α*-trihydroxy-5*β*-methyl cholanate) may be prepared as described by Fieser and Rajagopalan [4], or more simply, by dissolving 50 g of the acid in 150 ml of methanol and adding 5 ml of concentrated hydrochloric acid. This solution is refluxed for 15 min in a covered beaker and allowed to stand overnight in the refrigerator. The yield is about 95 %. The mother liquor is discarded. Recrystallization from methanol does not significantly improve the purity of the product.

Diacetoxy-12α-hydroxy-methyl cholate, III (3*α*,7*α*-diacetoxy-12*α*-hydroxy-5*β*-methyl cholanate) is prepared according to the revised method of Fieser and Rajagopalan [5]. Methyl cholate (50 g) is dissolved in a mixture of 100 ml of dioxane and 100 ml of pyridine with warming. When the clear solution has cooled to room temperature, 150 ml of acetic anhydride is added. After 20 h at room temperature, 200 ml of water is added with thorough mixing. Crystallization occurs within minutes. The reaction mixture is kept in the refrigerator overnight and filtered. The yield is about 50 %; recrystallization is performed from methanol.

3,7-Diacetoxy-12-keto-methyl cholate, IV (3*α*,7*α*-diacetoxy-12-keto-5*β*-methyl cholanate) is prepared by chromic acid oxidation in acetone as described by Sato and Ikekawa [8]. 3*α*,7*α*-Diacetoxy-12-hydroxy-5*β*-methyl cholanate (25 g) is dissolved in 250 ml of acetone with warming. After cooling, Kiliani's solution (53 g of chromium trioxide and 80 g of concentrated sulfuric acid are dissolved in 400 ml water) is added slowly while the solution is stirred continuously. After an orange-green color persists for 15 min, a little water is added so that the chromium dioxide separates into a viscous aqueous phase on the bottom. The upper acetone phase is decanted into a very large vessel and the lower phase is carefully washed once or twice with small volumes of acetone. The pooled acetone phases are neutralized with sodium hydroxide. A large excess of water is added with stirring. The 3*α*,7*α*-diacetoxy-12-keto-5*β*-methyl cholanate crystallizes instantaneously; the yield is quantitative. The crystals are separated by filtration and washed thoroughly with water; recrystallization is unnecessary.

12-Keto chenodeoxycholic acid, V (3*α*,7*α*-dihydroxy-12-keto-5*β*-cholanic acid) is prepared by saponifying the diacetoxy-12-keto-methyl cholanate overnight at room temperature in 10 % ethanolic potassium hydroxide (10 ml of a 1 g/ml solution of KOH is added to 90 ml absolute ethanol). After acidification of the solution and removal of the ethanol on a rotary evaporator, the free acid can be extracted into ether, then crystallized easily from aqueous ethanol. Indeed, the acid can be crystallized directly from the saponification reaction mixture by acidification, warming, and trituration with ethanol.

Chenodeoxycholic acid, VI (3*α*,7*α*-dihydroxy-5*β*-cholanic acid) is prepared by Wolff-Kishner reduction (Huang-Minlon modification) of the preceding product; the reaction may be scaled down considerably without effect on the yield. 20 g 3*α*,7*α*-dihydroxy-12-keto-5*β*-cholanic acid is placed in a three necked round bottom flask. To this is added 300 ml ethylene glycol and a solution of 30 g KOH in 60 ml water. 30 ml 85 % hydrazine hydrate, or its equivalent in 64 % hydrazine hydrate, is added and the reaction mixture

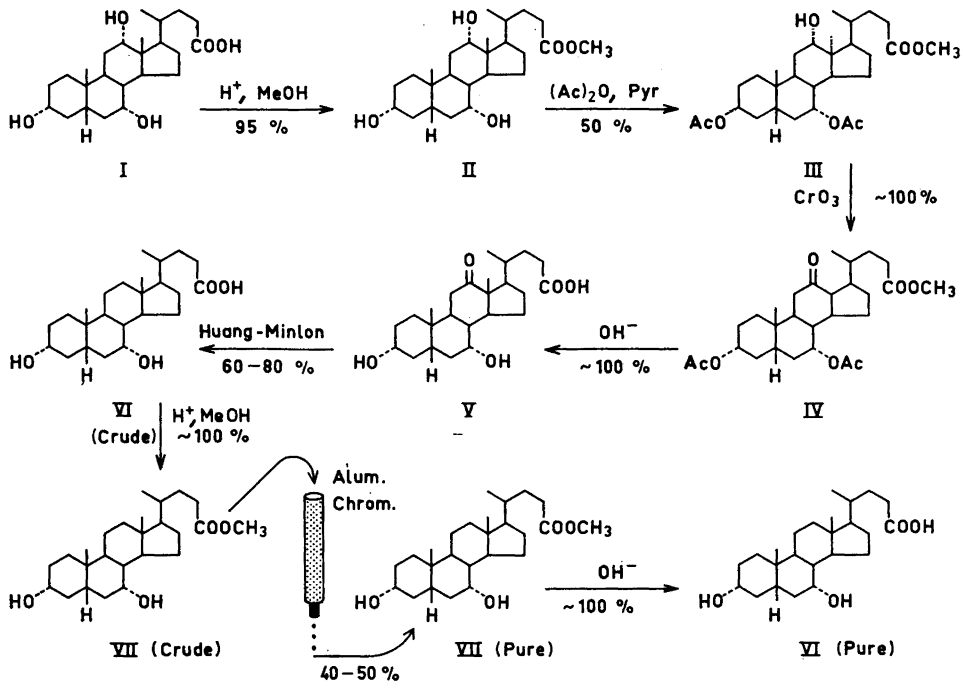

Fig. 1. Scheme for preparation of chenodeoxycholic acid from cholic acid. The yield of each step is indicated. The overall yield is 10–15 %. A modification of this scheme, in which the complete saponification of IV to yield V is omitted, is described in the text.

kept at 100° for 2 h under reflux. The heat is then increased, a few chips of porcelain are added, and the hydrazine is boiled off. The temperature of the reaction mixture is brought to 185–190° as the hydrazine is removed, and held there for 3–4 h under reflux. The solution is then allowed to cool, diluted with a large excess of water, and acidified slowly and with continuous shaking to pH 3. The crude acid is allowed to precipitate for 15 min or so, and then filtered. The filter cake is washed throughly with water and transferred to a beaker; the yield at this point is usually 70–80 % with respect to the keto-compound.

The crude acid is dissolved in about 200 ml of ether:benzene, 3:1 v/v, and transferred with additional washes of the same solvent mixture to a separatory funnel. The upper phase is washed throughly with water; the yellow oily precipitate accumulating at the interface is discarded, and the upper phase poured into a flask and dried with sodium sulfate. Thin layer chromatography at this point using the solvent system for free bile acids [10] will show six or seven components. The major spot is chenodeoxycholic acid; there are two or three less polar and two or three more polar impurities. The crude acid will form a gel when dissolved in a small amount of hot ethyl acetate which is allowed to cool slowly. Its m.p. after drying will be about 133–140°.

Methyl chenodeoxycholate (3α,7α-dihydroxy-5β-methyl cholanate VII) is prepared from the crude acid by refluxing a solution of the acid in methanol containing 0.02 volumes concentrated hydrochloric acid for 15 min and allowing to stand overnight at room temperature. The methanol solution is concentrated on a rotary evaporator, poured into a large excess of ether and the ether phase washed repeatedly and rapidly with small volumes of water until the washings are neutral. The ether phase is dried with sodium sulfate, filtered, and evaporated; the crude methyl ester is dissolved in a small volume of benzene

Chromatographic purification. A column of alumina (activity level III) is prepared in benzene as described by Neher [14]. The sample is applied to the column; for each 100 g of adsorbent, 2.5 g crude methyl chenodeoxycholate can be chromatographed satisfactorily. Elution is begun with ethyl acetate-benzene (10/90, v/v) and continued with stepwise increments of 5 % in the ethyl acetate concentration. Generally for a column of 200 g of alumina, 200 ml of a given eluant concentration is sufficient. Small fractions, *e.g.* 25 ml, are collected in weighed flasks and rapidly evaporated with a rotary evaporator. The fractions are then weighed and dissolved in sufficient ethyl acetate to give a concentration of 20 mg/ml. 5 μl of each fraction is then examined by thin layer chromatography on 66 × 66 mm glass plates [11]; ethyl acetate-cyclohexane (1:1, v/v), is used as developer and a standard is run. The R_F of methyl chenodeoxycholate is about 0.45. The spots are detected with 10 % ethanolic phosphomolybdic acid; a chromatographic run requires about 10 min. With 25 % ethyl acetate in benzene, a slightly less polar impurity is eluted. It generally tails into the methyl chenodeoxycholate, and it is often necessary to repeat elution with this concentration. The methyl chenodeoxycholate is eluted with 30 % ethyl acetate in benzene. The early fractions are slightly contaminated by the less polar impurity; subsequent fractions show no detectable impurity by thin layer chromatography. Elution is continued with 35 % or 40 % ethyl acetate in benzene until the fractions contain negligible ester. The more polar impurities are generally not eluted. Those fractions containing the pure methyl ester are pooled and the solvent removed on a rotary evaporator; the methyl ester may be crystallized easily by dissolving in a small volume of hot benzene, adding three volumes of hot heptane, and allowing the clear solution to cool slowly. Fine needles are obtained, m.p. 90–91° after drying. The impure fractions which contain predominantly methyl chenodeoxycholate, are pooled and saved for re-chromatography.

Chenodeoxycholic acid VI, (3α,7α-dihydroxy-5β-cholanic acid) is prepared by saponifying the pure methyl ester in 5 % ethanolic potassium hydroxide overnight at room temperature. Following neutralization to pH 8–9, the alcohol is removed with a rotary evaporator; the solution is acidified to pH 3, the acid extracted into ether and washed well with water. Following drying with sodium sulfate, filtration, and evaporation of its ether solution, the acid is dissolved in a small volume of hot ethyl acetate. About two volumes of hot heptane are added, an amount considerably below that inducing turbidity. The clear solution is allowed to cool slowly to room temperature. Masses of "cottony" needles appear without seeding; the first crystallization contains about 90 % of the yield from the column (Fig. 2). The m.p. is 119° after drying overnight in a vacuum pistol over phosphorus pentoxide. Generally, but not always, it is possible to obtain a structured gel rich in solvent by dissolving the pure acid in a small volume of hot ethyl acetate alone and allowing the solution to cool quite slowly. On filtration, this aggregrate loses a great deal of solvent. On prolonged drying, it becomes a glass, m.p. 146. The final yield is 10–15 % relative to cholic acid. The yield from the column is 40–50 % relative to the crude methyl ester.

The purity of the final preparation is assayed by TLC. 1, 5, 10, 50, 100 and 200 μg samples are applied to a 20 × 20 cm plate; after developing, the plate is heated to 160–180° and then sprayed while still hot with 10 % ethanolic phosphomolybdic acid [15]. Only one spot is seen. If impurities are present, a semiquantitative estimate of their amount can be made.

Sodium taurochenodeoxycholate (Sodium 3α,7α-dihydroxy-5β-cholanyl-taurate). The conjugation step is carried out as described by Norman [12]. Equimolar amounts of chenodeoxycholic acid and tri-butylamine are dissolved in dioxane (previously dried with molecular sieves). Additional molecular sieves are added. The solution is cooled to 10° and a 5 % excess in mol. prop. of ethyl chlorocarbonate is added. The solution is held at 10° for at least a half hour. Sodium taurate is prepared by dissolving taurine in the equivalent amount of 1 N NaOH. A 25 % excess in mol. prop. of sodium taurate is added to the dioxane solution with shaking, and the reaction mixture is removed from the cooling bath. The reaction mixture is allowed to stand for 30 min or so at room temperature, then evaporated without warming to near dryness using a rotary evaporator. A large volume of chloroform-methanol, 2:1, v/v, is added plus a little absolute alcohol if necessary to give one phase. After standing for 30 min, the insoluble taurine and sodium chloride are removed by filtration.

The solution is again evaporated without warming to near dryness and dissolved in a small volume of 95 % ethanol. It is then percolated slowly through a cation exchange column (Dowex 50-W, X2, H^+ form, prepared in 95 % ethanol) to remove the tri-butylamine. The alcoholic solution is neutralized to a pH of 3—4 (not higher) with sodium ethoxide (freshly prepared using spectroscopic grade ethanol) and after standing for some minutes, refiltered if necessary.

The solution is again evaporated without warming to dryness and dissolved in water. Any excess of free acid is evidenced by turbidity. If present the aqueous solution is extracted once or twice with ethyl acetate, then twice with small volumes of ether. The aqueous solution is then percolated through a second cation exchange column (Dowex 50—W, H^+ form, prepared in water) and extracted rapidly with ethyl acetate [6], then twice with small volumes of ether. The solution is neutralized to pH 5 with freshly prepared sodium hydroxide, and then briefly placed on a rotary evaporator to remove the remaining traces of ether. The clear aqueous solution, now free of solvent, unconjugated bile acid, and mineral salts, is freeze dried. A white, free flowing powder is obtained, m.p. 182—184°. If all evaporation steps have been performed without warming the reaction mixture, the product will be completely white and will not discolor with prolonged drying *in vacuo* at 100°. The final product dissolves readily in water or chloroform-methanol, 2:1, v/v, to give a clear solution without residue. The yield is 60—80 %. The assay of purity is carried out by thin layer chromatography as described above; the solvent system for conjugated bile acids [10] is used. Generally, a chromatographically pure product is obtained.

Sodium glycochenodeoxycholate (Sodium 3α,7α-dihydroxy-5β-cholanyl glycinate). The conjugation step is carried out as described by Norman [12]. Equimolar amounts of chenodeoxycholic acid and tri-butylamine are dissolved in dioxane (previously dried with molecular sieves). Additional molecular sieves are added. The solution is cooled to 10° and a 5 % excess in mol. prop. of ethyl chlorocarbonate is added. The solution is held at 10° for at least a half hour. Sodium glycinate is prepared by dissolving glycine in the equivalent amount of 1 N NaOH. A 10 % excess in mol. prop. of sodium glycinate is added to the dioxane solution with shaking, and the reaction mixture is removed from the cooling bath. The reaction mixture is allowed to stand for 30 min or so at room temperature and then concentrated without warming to a syrup, using a rotary evaporator. The syrup is then dissolved in pre-equilibrated lower phase of a two phase system of water-ethanol-diethyl ether-petroleum ether, (1:1:1:1, v/v [17]) and the pH is adjusted to 9—10 with sodium hydroxide. The alkaline lower phase is extracted three times with pre-equilibrated upper phase of this system. It is convenient to use a separatory funnel and remove the upper phases by a capillary pipette attached to a water suction line. The alcoholic lower phase is then adjusted to pH 6 and extracted several times with small volumes of the upper phase of this system. The alcohol is then removed from the lower phase by careful evaporation without warming the solution to more than 37°. The aqueous solution is acidified to pH 5 and again extracted with the upper phase of this system. Thin layer chromatography of the lower phase should show not more than a trace of the unconjugated acid.

The solution is then acidified to pH 3 and extracted into ether-ethyl acetate (2:1, v/v). The ether-ethyl acetate phase is washed well with water and dried briefly in the separatory funnel with sodium sulfate. It is then filtered, and evaporated to dryness without warming. The white gum is dissolved in a small volume of methanol and neutralized to pH 8 with freshly prepared sodium hydroxide. The methanol is removed by evaporation, again without warming, and the residue is dissolved in a small volume of water. The clear aqueous solution is freeze dried. The final product is a white, free flowing powder. Yields are 60—80 %. When the product is examined by thin layer chromatography it will not be completely pure, being contaminated by a more polar impurity as well as free chenodeoxycholic acid. Both impurities are present to 1—3 %.

Although the procedure is laborious, a chromatographically pure product may be obtained by adsorption chromatography. Silicic acid is inactivated with water, 35 g water per 100 g silicic acid [18]. 40 g Celite per 100 g silicic acid is added to improve the flow rate: a column is prepared in benzene. The glycochenodeoxycholic acid is methylated as described for the methylation of chenodeoxycholic acid. The methyl glycochenodeoxycholate is dissolved in warm benzene-ethyl acetate (95:5, v/v) and applied to the column.

Chromatography is carried out as described for the purification of methyl chenodeoxycholate. Any contaminating methyl chenodeoxycholate is eluted with 25—30 % ethyl acetate in benzene. Methyl glycodeoxycholate is eluted with 40—45 % ethyl acetate in benzene. The more polar impurity remains on the column. Saponification is carried out at room temperature for 24 h using absolute ethanol-0.2 N NaOH, (1:1, v/v). Saponification is complete and there is no hydrolysis of the peptide bond. After acidification of the saponification liquor, the acid is extracted into ether-ethyl acetate (2:1, v/v) which is washed well with water. The subsequent preparation of the sodium salt is carried out as described.

DISCUSSION

Preparation of the crude acid. The bile acid intermediates in the synthesis described have been purified by recrystallization and/or column adsorption chromatography, until they were chromatographically pure by TLC. Their m.p.'s after drying, the highest m.p.'s recorded in the literature, and their approximate R_F's, both absolute and relative, in several thin layer chromatographic systems are shown in Table 1. In practice the intermediates in the synthesis described are usually purified by a single recrystallization. They show traces of impurities when examined by TLC and their m.p.'s are usually a degree or so below those recorded for the pure compounds. In view of the extensive final chromatographic procedure, thorough purification of each intermediate seems unnecessary. Furthermore, Wolff-Kishner reduction of chromatographically pure 12-keto-chenodeoxycholic acid resulted in a raw acid contaminated by at least five impurities.

The 3α,7α,-diacetoxy-12-keto-5β-methyl cholanate was hydrolyzed quite easily. Complete saponification occurred at room temperature in 10 % ethanolic potassium hydroxide; saponification with the same base concentration at 80° resulted in an identical product but with marked darkening of the saponification mixture. Crystallization of the resultant 12-keto-chenodeoxycholic acid occurred easily when an amorphous suspension in aqueous alcohol was heated on a water bath. Comparison of the behaviour of 3α,7α-diacetoxy-12-keto-5β-methyl cholanate and of that of 3α,7α-diacetoxy-5β-methyl cholanate under extremely mild saponification conditions showed the latter compound to be much more resistant to hydrolysis of the 7α-position, in agreement with the observations of Hauser *et al.* [7]

If the Wolff-Kishner reduction was carried out as described on 3α,7α-diacetoxy-12-keto-5β-methyl cholanate instead of 3α,7α-dihydroxy-12-keto-5β-cholanic acid, the yield was much smaller and a great deal of insoluble brown material was formed. If the extraction procedure described was used, a rather pure crude acid was nonetheless obtained. The much more satisfactory reduction obtained with 12 keto-chenodeoxycholic acid may be related to some extent to the apparent influence of the 12-keto group on the ease with the 7α-acetoxy group is saponified. It was furthermore observed that the Wolff-Kishner reduction proceeded smoothly with excellent yields if the 3α,7α-diacetoxy-12-keto-5β-methyl cholanate was merely suspended in ethylene glycol and KOH, the solution warmed slightly for complete wetting of the crystals, and the suspension allowed to stand two days before proceeding with the reduction step; this procedure was consistently satisfactory and may be used instead of the reaction scheme described.

Table 1. Melting points and thin layer chromatographic behaviour of compounds involved in the preparation of chenodeoxycholic acid from cholic acid. The compounds had been purified by column (adsorption) chromatography and were estimated to be at least 99.5 % pure. The observed melting points denoted by § were values observed after the samples had been kept some months at room temperature in stoppered bottles. The figures in parentheses after the reported melting points indicate the appropriate references.

Thin layer chromatography was performed on 20 × 20 cm glass plates using Kieselgel G as adsorbent. The plates had been activated for 30 min at 120° about 2 h before the application of the samples, and in the interval were stored in a desiccator over silica gel. The chromatography jars contained a wide band of filter paper along one wall. Solvent mixtures were poured into the chromatography containers which were then closed and allowed to equilibrate for 2 1/2 hours before the chromatographic run. The plates were placed with as little disturbance of the container's atmosphere as was possible. The chromatographic path was 10.0 cm. R_F and $R_{Cholesterol}$ (R_{Chol}) values are expressed to the closest 0.05 because of their lack of precision.

The compositions of the solvent systems (volume/volume) were: A. ethyl acetate, 30; heptane, 70; and acetic acid, 1. B. ethyl acetate, 15; cyclohexane, 85; and acetic acid, 1. C. ethyl acetate, 40; benzene, 60; and acetic acid, 1. D. benzene, 10; propanol, 10; isoamylacetate, 40; di-isopropyl ether, 30; carbon tetrachloride, 20; and acetic acid, 5.

Compound	Crystallized from	Melting points		Thin layer chromatographic behaviour with solvent system							
		Observed	Reported	A		B		C		D	
				R_F	R_{Chol}	R_F	R_{Chol}	R_F	R_{Chol}	R_F	R_{Chol}
3*a*,7*a*,12*a*-Trihydroxy-5*β*-methyl cholanate (II)	Methanol	152–155	156–157 (4)	0	0	0.10	0.15	0.05	0.05	0.10	0.15
3*a*,7*a*-Diacetoxy-12*a*-hydroxy-5*β*-methyl cholanate (III)	Methanol	187.4–187.5	187–188 (4)	0.10	0.35	0.35	0.60	0.50	0.85	0.75	0.95
3*a*,7*a*-Diacetoxy-12-keto-5*β*-methyl cholanate (IV)	Methanol	180.0–180.5	177–179 (4) 178–179 (8) 176–178 (7) 184–186 (from acetone (7)	0.15	0.45	0.40	0.70	0.65	1.05	0.80	1.00
3*a*,7*a*-Diacetoxy-5*β*-methyl cholanate	Methanol-water	129.8	129–130.5 (8) 130–132 (7)	0.30	0.95	0.60	1.00	0.75	1.20	0.85	1.10
3*a*,7*a*-Diacetoxy-12-thioketal-5*β*-methyl cholanate	Methanol	178.3–178.9	175–176 (8)	0.20	0.65	0.55	1.00	0.75	1.20	0.90	1.15
3*a*,7*a*-Dihydroxy-5*β*-methyl cholanate (VI)	Benzene-heptane	90–91, 82–85 §	80–94 (6, 7) 85–94 (7)	0	0.05	0.15	0.25	0.15	0.20	0.40	0.55
3*a*,7*a*-Dihydroxy-5*β*-cholananic acid (VII)	Ethyl acetate-heptane	119 115–117 § 146 (gel)	145–146 (8) 120–130, 125–145, 145–148 (7)	0	0	0.10	0.20	0.05	0.10	0.35	0.50
3*a*,7*a*-Dihydroxy-12-keto-5-cholanic acid (V)	Ethyl acetate	221–222	219–220 (25)	0	0	0.05	0.10	0	0.05	0.25	0.30
Cholesterol	—	—	—	0.35	1.00	0.60	1.00	0.60	1.00	0.80	1.00

The modification of the Wolff-Kishner reduction developed for alkali sensitive keto-groups [19] was also tried; higher yields were obtained. However, one of the major impurities in the raw acid had an R_F only slightly greater than that of chenodeoxycholic acid when examined by TLC using ethyl acetate-benzene as developer. It could be predicted that a column chromatographic separation of this component from chenodeoxycholic acid would be difficult.

In the method of Sato and Ikekawa [8], the 12-thioketal-derivative is prepared by a boron trifluoride catalyzed reaction with ethane dithiol. Subsequent desulfuration and saponification yield chenodeoxycholic acid in high yields. This procedure was abandoned because of the inability to prepare the 12-thioketal-derivative quantatively. This meant that the final product was contaminated by 12-keto-chenodeoxycholic acid. Occasionally a non-identified less polar impurity was also noted. Both of these substances co-crystallied with chenodeoxycholic acid. The formation of mixed crystals of 12-keto-lithocholic acid (3α,hydroxy-12-keto-5β-cholanic acid) with chenodeoxycholic acid is well documented [20]. The 12-thioketal-derivative could be purified by adsorption chromatography on alumina; see Table 1. The subsequent steps of desulfuration and saponification proceeded smoothly. However, for an absolutely pure product, it was necessary to methylate the final product and rechromatograph it as described. Because of the undesirability of two chromatographic purification steps as well as the offensiveness of the odor of the ethane dithiol, we chose to employ the Wolff-Kishner reduction. A sample of chenodeoxycholic acid, m.p. 142—144°, synthesized by this method and kindly supplied by Dr. Y. Sato, was judged to be 95—96 % pure by TLC; both more polar and less polar impurities were present.

The method of Hauser, Baumgartner and Meyer [7] is quite similar to that described except that is more elaborate. A sample of chenodeoxycholic acid, m.p. 142—146°, synthesized by that method and kindly supplied by Professor Kuno Meyer, was at least 99 % pure by TLC, containing less than 1 % of a less polar impurity.

Adsorption chromatography of the crude methyl ester. The chromatographic procedure described was consistently satisfactory, although the resolution of some columns was better than others. The slightly less polar impurity invariably trailed into the methyl chenodeoxycholate peak; usually the first one fourth or fifth of the peak had to be saved for rechromatography. In the examination of the fractions, 100 μg of each sample was applied to the plate. As the detecting method used easily revealed 1 μg, any sample appearing chromatographically homegeneous had a purity of at least 99 %. Silicic acid, equilibrated with 10 % water by weight [18] was also used for the column chromatographic purification of crude methyl chenodeoxycholate. An identical product was obtained, but the yield of chromatographically pure methyl chenodeoxycholate was appreciably smaller.

The final, chromatographically pure acid crystallized easily as described. Such crystallization usually purified the acid in that the less polar components, present at most to 1 %, remained in the mother liquor; so also did a faint yellowish color occasionally acquired during the final saponification procedure.

Attempts were made to abbreviate the chromatographic procedure by pouring the crude methyl ester dissolved in ethyl acetate-benzene (40:60, v/v)

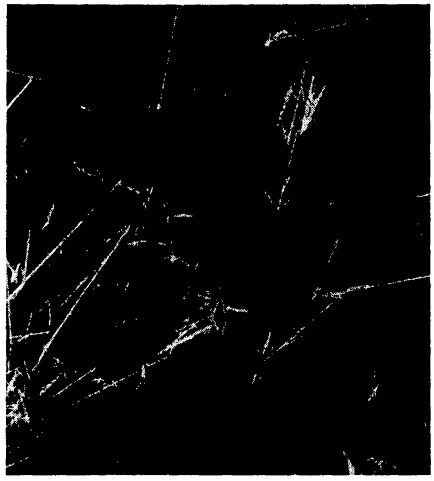

Fig. 2. Photograph of crystals of chenodeoxycholic acid, m.p. 119° form. The sample had been crystallized from ethyl acetate-heptane.

over an alumina column (activity level III) prepared in the same solvent mixture. The eluant was completely free of more polar impurities, but complete purification could not be obtained by crystallization, as a few percent of the less polar impurities were always present in the crystals. The method was of interest, however, in demonstrating an extremely rapid and convenient means of preparing 97—98 % pure chenodeoxycholic acid.

Characteristics of chenodeoxycholic acid, m.p. 119°. When the crystals of chromatographically pure chenodoxycholic acid were observed to have a m.p. of 119° (after drying in vacuo at 100°) it was not appreciated that a polymorphic form of this bile acid had been prepared. Rather, two possibilities were considered, in view of the known property of many bile acids to form mixed crystals with similar compounds [20,21]: either the m.p. 119° compound was a mixed crystal of two more compounds, or all previous preparations of this acid with m.p. 143—146° were mixed crystals of two or more components. Both possibilities seemed extremely unlikely.

The m.p. 119° compound had no detectable impurities when examined by bidimensional TLC using solvent system D (Table 1) or ethyl acetate-cyclohexane-acetic acid (50:50:2, v/v) as developers and concentrated nitric acid-concentrated sulfuric acid-water (3:4:3, v/v) as detecting agent. Gas chromatographic analysis [22], kindly performed by Dr. J. Gürtler of the Clinical Chemistry Department, Lund Hospital, showed the compound to be free of volatile impurities and to have an identical retention time to that of authentic chenodeoxycholic acid, prepared according to Fieser and Rajagopalan [4] and to chenodeoxycholic acid isolated from human bile. Elemental analysis, performed by the Division of Analytical Chemistry, University of Lund was satisfactory. (Found: C 73.2; H 10.3. Calc. for $C_{24}H_{40}O_4$: C 73.4; H 10.3). Its optical rotation, $[\alpha]_D^{20} = +11.5 \pm 2°$ (c, 1 in dioxane) agreed with published values [4,7,8]. Infra-red analysis, kindly performed by Dr. I. Fischmeister of the Karolinska

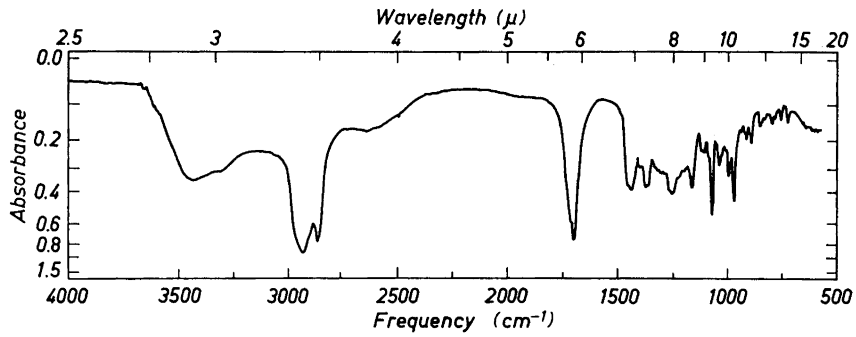

Fig. 3. Infrared spectrum of chenodeoxycholic acid, m.p. 119° form. The spectrum was recorded with a Perkin-Elmer Model 21 spectrophotometer equipped with a sodium chloride prism. A pellet of 2.07 mg substance and 300 mg potassium bromide was used.

Institutet, revealed essentially the same spectrum as that of authentic chenodeoxycholic acid (Fig. 3). Chromatography of the impure methyl chenodeoxycholate on partially inactivated silicic acid as described, instead of alumina, yielded an identical product, m.p. 119°.

Attempts to change the m.p. of the 119° form by heating 24 h at 130° were unsuccessful; an amorphous glass, m.p. 129°, was obtained. Recrystallization of the m.p. 119° form from acetic acid-heptane-ether [7] yielded needles, m.p. 119°. It seemed unlikely that the impurities in the samples of authentic chenodeoxycholic acid supplied by Professor Kuno Meyer and Dr. Y. Sato could be responsible for the disparity in melting points.

The matter was finally resolved in a number of ways. A sample of unpurified chenodeoxycholic acid which had been prepared by Sarel and Yanuka's modification [19] of the Wolff-Kishner procedure was carefully cooled in ethyl acetate-heptane. Two solid forms, differing completely in appearance were obtained. The first, large prisms, had a m.p. of 113—121°. The second, amorphous pieces, had a m.p. of 137°. The two forms were carefully separated manually, chromatographed, and found to have an identical composition. It was apparent therefore that two polymorphic forms of slightly impure chenodeoxycholic acid existed.

That pure chenodeoxycholic acid also possessed two polymorphic forms was proved by recrystallizing from ethyl acetate-heptane the sample of authentic chenodeoxycholic acid supplied by Dr. Y. Sato. Feathery prisms, m.p. 119—120° before drying and 119° after drying were obtained. A portion of the sample of Professor Kuno Meyer's had been dissolved in ethyl acetate for use as a chromatography standard; this was kept in a loosely capped bottle and the solvent accidentally evaporated, leaving fine crystals, m.p. 117°.

Finally, a 1.5 g preparation of chromatographically pure chenodeoxycholic acid was "crystallized" from ethyl acetate. In this solvent, the acid did not actually crystallize, but formed a structured gel rich in solvent (*cf.* Refs. [6,7]). On filtration, the gel lost much solvent and collapsed to an amorphous powder. This powder, on drying, formed a glass, m.p. 146°. The mother liquor was

rewarmed, hot heptane added, and a single seed of m.p. 119° chenodeoxycholic acid added. Within minutes, masses of rosettes appeared which after drying had a m.p. of 119°. TLC of the two forms showed each to be completely pure chenodeoxycholic acid.

It is curious that this crystalline form, m.p. 119°, of chenodeoxycholic acid has not been prepared previously. Extremely impure chenodeoxycholic acid forms a gel in ethyl acetate as described. Its m.p. after drying is about 133—137°. As the acid is purified, this type of aggregration persists, but the m.p. of the dried solid rises to 143° and even to 146° or 148°. The higher m.p. aggregrate can, however, be obtained with rather impure acids. A sample of chenodeoxycholic acid prepared in this laboratory some years ago and not more than 85 % pure by TLC had a m.p. of 141°. In contrast, it is quite rare to observe the crystalline form, m.p. 119°, unless the acid is quite pure. That a true crystalline form of chenodeoxycholic acid with m.p. 140—146° can be prepared is well documented, however, even if gel formation is more common. Both Fieser and Rajagopalan [4] and Sato and Ikekawa [8] observed prisms.

The m.p. of the polymorphic form reported here is usually 119° but several chromatographically pure samples have had m.p. from 112—120° or 115—117°. At these respective temperatures, the crystals often fuse to a glass which does not completely melt until about 145°. In view of this observation as well as that of Hauser *et al.* [7], who crystallized chenodeoxycholic acid with a m.p. of 125—145°, as well as 120—130°, determination of the melting point seems a rather unsatisfactory method for characterization of this compound.

The preparation and properties of the taurine and glycine conjugates

Sodium taurochenodeoxycholate. The method described for the preparation of this taurine conjugate is based on extensive experimentation to find the simplest, most rapid method for preparing pure taurine conjugates using the method of Norman [12]. The method can be applied equally well to the preparation of sodium taurocholate, sodium taurodeoxycholate or sodium alkyl taurates. Chloroform-methanol (2:1, v/v) is an excellent solvent for removing sodium chloride; it is much more convenient to use than 90 % ethanol.

The Dowex 50-W must have been extensively washed with alkali and acid before use or it will discolor the bile acid solution. After the alcoholic solution has passed through the ion exchange column, it should not be neutralized to a pH higher than 3—4. Otherwise when the reaction mixture is dissolved in water, the free bile acids will remain in solution forming mixed micelles with the conjugate anions. Satisfactory extraction will be difficult, and if the aqueous solution containing an appreciable amount of free bile acid is poured over the ion exchange column, the free acid will precipitate out of solution clogging the column.

All evaporations should be performed without warming the reaction mixture to more than about 37°. If it is heated during evaporation, discoloration frequently occurs which is impossible to remove completely. It is advisable to do an entire synthesis during a single day, as the reaction mixtures may discolor on prolonged standing.

The taurine conjugates were generally not isolated by Norman [12] in the sulfonic acid form. Taurochenodeoxycholic acid can be prepared by pouring the aqueous solution slowly through a cation exchange column and freeze drying the effluent. On one occasion, the free acid crystallized from water, m.p. (after drying *in vacuo* at 100°) 188°, with apparent decomposition.

Crystallization of the trihydroxy- and dihydroxy-taurine conjugated bile acids as their sodium salts is extremely difficult. They do not readily crystallize from any of the common anhydrous solvents. They are best crystallized from 86—90 % ethanol-ether or 86—90 % ethanol-ethyl acetate mixtures [12,16] but the procedure is time-consuming and often unsatisfactory. When crystallization can be done, however, it is a powerful purification step as contaminating free acids and pigments generally remain in the crystallization liquor. The taurine conjugates can also be precipitated from ethanolic solution by the addition of ether. The precipitate is usually extremely hygroscopic.

Freeze drying gave a product with excellent characteristics that was not hygroscopic. The method is applicable only if all impurities have been removed previously.

Sodium glycochenodeoxycholate. All glycine conjugates prepared by the method of Norman [12] contain a more polar impurity. This polar impurity is an acid and possesses the same chromatographic mobility as the major product obtained when the conjugation step is performed with glycyl-glycine instead of glycine. The impurity, present to 2—8 % in the crude preparation, may well be a glycyl-glyco-conjugate. In the preparation of glycine conjugates, therefore, a smaller excess of glycine is used. All attempts to crystallize glycochenodeoxycholic acid, using chromatographically pure preparations, have failed. The acid can be obtained as an amorphous white powder from ethyl acetate, m.p. 81—105°. Indeed, glycochenodeoxycholic acid, the dominant dihydroxy-bile acid in man [1], has never been crystallized.

We have not succeeded in preparing a chromatographically pure sample of glycochenodeoxycholic acid using the liquid-liquid extraction scheme described. The finally products have generally been 95—97 % pure, containing 1—2 % of free chenodeoxycholic acid and 1—3 % of the more polar impurity. It is impossible to make an accurate estimate of the amount of impurity present by TLC because the color yield per μg of all these substances is unknown.

The adsorption chromatographic procedure on inactivated silicic acid may be preferable to the reversed phase partition chromatography schemes published by Norman [24] for the purification of conjugated acids. The procedure described is much simpler and the columns have a considerably larger capacity. Methyl glycodeoxycholate could not be eluted from alumina columns.

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