The physiological and industrial importance of cholic and desoxycholic acids, the two main constituents of the bile acids, makes very desirable a method for their qualitative separation and, if possible, for their quantitative determination. The difference in their solubilities in various solvents or of the solubilities of the salts used in their preparation (1) is not very marked and causes losses too large to allow more than a rough estimation of their quantities and necessitates relatively large amounts of the starting material for successful operation.

The chromatographic analysis of these acids promised a successful separation. The difficulty of the method consists in the fact that the bile acids are colorless substances, and for the purpose of visual chromatographic separation they have to be converted into colored derivatives.

In the case of the bile acids which contain hydroxyl groups there is the possibility of converting them into colored compounds by esterifying the alcoholic OH groups by means of azoyl chloride (2). Although this method may prove successful, it seems to us more desirable to place the "chromophoric" group on the COOH group for the following reasons. (1) It is well known that the different OH groups of the bile acids react differently on esterification; so that without special precautions there may be formed partly esterified compounds in the case of the dihydroxyl and polyhydroxyl compounds which would complicate the chromatographic picture. (2) By coupling the "chromophoric group" to the carboxylic group, not only bile acids containing alcoholic hydroxyl groups, but also acids devoid of them, like cholenic and polyenic acids, as well as the ketonic derivatives, can be included in the scope of the method. (3) The alcoholic hydroxyl groups are known to cause strong adsorptivity of a compound (3), and therefore it is to be expected that mono-, di-, and polyhydroxyl compounds can be differentiated in the chromatogram of their mixture.

As a suitable chromophoric reagent for the esterification of the COOH group we have chosen the \( \omega \)-bromo-\( p \)-methylazobenzene. This substance is relatively simple and, being a derivative of benzyl bromide, possesses a highly reactive bromine atom which reacts under suitable conditions with the sodium salts of acids, giving a yield of 85 per cent or higher of the corresponding ester.
ω-Bromo-p-methylazobenzene was prepared by Burns, McCombie, and Scarborough (4) by bromination of 4-methylazobenzene but with very moderate yields. We found that this compound can be prepared with better yields and in a high state of purity by condensation of p-aminobenzyl alcohol with nitrosobenzene and the transformation of the hydroxymethyl compound into the corresponding bromine derivative by action of phosphorus tribromide, as described in the experimental part.

For the chromatographic separation of the colored esters we tried first activated alumina, silica gel, calcium carbonate, and magnesium carbonate. The two first were too active and did not allow a proper development of the chromatogram; the third was too weak in its adsorptive properties. MgCO₃ (a commercial preparation of the Corona Chemical Company, Sydney) proved a very satisfactory adsorbent for the colored esters dissolved in a mixture of benzene and petroleum ether (65–80°C) 1:2. For the development of the chromatogram we first use a mixture of benzene and petroleum ether 1:1 and then pure benzene. The ester of the cholic acid is adsorbed on top as an orange-colored band; the desoxycholic ester (if in mixture with cholic acid ester) is placed below and is lighter in color. It is possible to develop a narrow, almost white, band between these two bands of esters and with some practice there is no difficulty in a fair separation of the two colored zones, provided that a properly packed column is used so that there are no excessive irregularities in the formation of the bands.

5 per cent of cholic acid in desoxycholic acid and 10 per cent of desoxycholic acid in cholic acid can be easily discerned in the developed chromatogram.

At present we are engaged in extending this separation to a quantitative colorimetric determination of the proportions of the two acids present in the mixture and in the visual separation of the esters by using different chromophoric groups, e.g. C₆H₅·N=N·C₆H₄·CO·CH₂ halide.

EXPERIMENTAL

ω-Hydroxy-p-methylazobenzene—The condensation of p-aminobenzyl alcohol with nitrosobenzene in alcoholic solution at room temperature proceeds very slowly and gives very low yields. The same condensation in acetic acid yields mostly a brick-red compound, of a very high melting point, which is sparingly soluble in the usual solvents and is not the expected azo compound. In a mixture of alcohol and acetic acid, however, the condensation proceeds smoothly.

5.85 gm. (0.055 mole) of nitrosobenzene are dissolved in a mixture of 40 ml. of alcohol and 14 ml. of glacial acetic acid with careful heating to 40–50°.
The clear green solution is cooled to 15° and 6.1 gm. of aminobenzyl alcohol are added with continuous shaking in six portions within 5 to 10 minutes. The solution turns reddish brown and the temperature rises to approximately 40°. The mixture is shaken for an additional 15 to 20 minutes, when crystallization sets in and it is left for 3 days at room temperature. After standing for a day in a refrigerator, the crystals are filtered and pressed on a Büchner funnel. A further crop can be obtained by diluting the filtrate with water. After drying in air, the material is extracted with warm ether in a continuous extractor (a brown insoluble material remains in the cone). The ethereal solution is evaporated to a small volume until an abundant crystallization sets in. An equal volume of petroleum ether (40–60°) is added, the mixture is cooled, and the orange crystals are filtered with suction and washed with a mixture of ether-petroleum ether. M.p. 139–140.5°; 8.2 gm. = 78 per cent of the theory. This material is pure enough for the next step. For analysis it was recrystallized from ether-petroleum ether (40–60°); m.p. 141.5°.

26.42 mg. C12H11ClN2; 71.12 mg. CO2; 13.82 mg. H2O
Calculated, C 73.59, H 5.66; found, C 73.41, H 5.35

ω-Bromo-p-methylazobenzene—To 6.4 gm. of 4-hydroxymethylazobenzene (0.03 mole) suspended in 150 ml. of dry benzene, 3.2 gm. (120 per cent of the theory) of PBr3 dissolved in 35 ml. of dry benzene are added within 10 minutes in five equal portions with constant shaking. The orange-red solution turns dark and a flocculent precipitate settles on the walls. After standing ½ hour at room temperature, with periodical shaking, the reaction mixture is decomposed by addition of water. The benzene is evaporated and the residue is extracted with small portions of ether until the last extract is only slightly yellow in color. The ethereal extracts are collected and evaporated to a very small volume. After addition of double the volume of a low boiling petroleum ether (40–60°) and standing in the refrigerator, the crystals are filtered with suction and washed. Orange-yellow crystalline powder, m.p. 111–112°; yield, 6 gm. = 72 per cent of the theory.

1 p-Aminobenzyl alcohol was prepared according to Fischer and Fischer (5) with the following changes: After removing the azo and azoxy compounds, the filtrate was evaporated under reduced pressure (preferably in a CO2 stream) and the solid residue was exhaustively extracted with ether. The ethereal solution was dried over Na2SO4, the ether evaporated, and the dark brown residue distilled in vacuo. B.p. 169–171°, 11 mm.; m.p. 64.5°. After recrystallization from benzene, the melting point was 65°. Yield, 11 to 12 gm. = 68 to 75 per cent of the theory, from 20 gm. of p-nitrobenzyl alcohol (6).

2 We propose for this compound the term azyl bromide.
By a further recrystallization from ether-petroleum ether the melting point can be raised to 114.5–115°C.

24.5 mg. gave 16.7 mg. of AgBr.

C₁₃H₂₃N₂ Br. Calculated, Br 29.1; found, Br 29.0

Preparation of Colored Esters of Cholic and Desoxycholic Acids—To a hot solution of 1 gm. of ω-bromo-p-methylazobenzene in 15 ml. of alcohol is added a hot solution of 1.4 gm. of sodium cholate (or 1.3 gm. of sodium desoxycholate) (slightly less than the calculated amount) in 5 ml. of water and the homogeneous mixture is boiled under a reflux for 6 to 8 hours. The alcohol is removed under reduced pressure, and the residue is taken up with benzene and washed with water. After evaporation of the benzene layer under a vacuum, the residue is dried in vacuo over P₂O₅ and paraffin. For the purification of the crude orange-red ester 100 mg. are dissolved in a mixture of benzene and petroleum ether (70–80°C) 1:2 and passed through a column of 25 to 30 gm. of MgCO₃. The height of the filled portion is about 26 to 28 cm., 15 mm. inside diameter. The MgCO₃ was dried for 1 to 1.5 hours at 120°C and sifted to pass a 100 mesh screen but not a 150 mesh screen. The chromatogram was developed by washing with 100 ml. of a mixture of benzene-petroleum ether 1:1 and 150 ml. of benzene. The cholic acid ester remained as an orange-colored band, 90 to 100 mm. long, at the top of the column. The desoxycholic acid ester behaved similarly, but the color of the band was more yellow and the band from the same quantity of material was rather wider, 100 to 120 mm. 8 to 10 mg. of 4-hydroxymethylazobenzene (m.p. 140–140.5°C) could be isolated from a yellow band which traveled down the column and could be easily separated from the acid esters by a wide white zone or washed into the filtrate. Into the first filtrate passed an unidentified red-colored material, approximately 5 mg. For the elution of the esters we used a mixture of alcohol and benzene 5:95.

In the same way we separated mixtures (40 to 80 mg.) of cholic and desoxycholic esters in the proportions of 1:1, 1:5, 5:1, 1:10, and 10:1.

In the developed chromatogram there was always an easily perceptible pale band on the border between the cholic and desoxycholic esters.

The chromatogram was eluted and the purity of the material separated was tested by the following procedure. The solution of the ester in alcohol containing benzene was evaporated under reduced pressure, the residue taken up in 1 to 1.5 ml. of alcohol, 1 to 2 drops of 50 per cent KOH were added, and the mixture was refluxed for 1 to 1.5 hours. Water was added and the alcohol was removed by repeated evaporation. The strong alkaline solution was acidified and the acids were redissolved by adding a solution of Na₂CO₃. The mixture was extracted three to four times with ether, until the ether layer was colorless, freed from the dissolved ether by heating,
cooled, and acidified to Congo red. The separated acid was filtered and dried at 120° over P₂O₅ at 8 to 10 mm. for 3 hours. The cholic acid fraction invariably had a melting point of 196–198°; that of desoxycholic acid was 171–172° (checked as the acetic acid-choleic acid, m.p. 142°).

For the purpose of the chromatographic analysis it is not necessary to submit the crude mixture after esterification to any purification, as the esters of the cholic and desoxycholic acids are much more strongly adsorbed on MgCO₃ than are the contaminants. The contaminants travel down the column or pass into the filtrate.

SUMMARY

Cholic and desoxycholic acids were transformed into colored esters by permitting their sodium salts to react with ω-bromo-p-methylazobenzene.

The yellow- to orange-colored esters were separated in a chromatogram by adsorption on magnesium carbonate.

The orange-colored cholic acid ester is more strongly adsorbed and is separated in the developed chromatogram from the lighter colored desoxycholic acid ester by a white band.

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