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Studies in the Bile Acids

1. A METHOD OF SEPARATION AND IDENTIFICATION

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In order to study the occurrence and metabolism of the bile acids, it is necessary to have methods of identifying the constituents of a mixture. Mixtures of bile acids are difficult to handle by classical techniques, which depend on the formation of characteristic crystalline derivatives. Frequently, even relatively pure compounds are not easy to crystallize, and large amounts of material are necessary if minor constituents are to be identified. As pointed out by Haslewood & Wootton (1950), much previous work is not based on acceptable experimental evidence.

In recent studies of steroid hormones and their metabolites, considerable use has been made of chromatographic separation combined with infra-red spectrometry (Dobriner, Lieberman & Rhoads, 1948*a*; Dobriner *et al.* 1948*b*; Lieberman & Dobriner, 1948; Jones & Dobriner, 1949). In combination, these techniques have provided a powerful method for separating and identifying the individual non-acidic steroids of complex mixture. It therefore seemed probable that a similar method could be applied to the bile acids.

A successful procedure is described here. Initial trials with pure substances showed that the methyl esters of the common bile acids are separable on silica gel. These esters are soluble enough to enable their specific infrared spectra to be recorded in carbon disulphide solution.

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EXPERIMENTAL

Solvents. All solvents except ether were of 'C.P.' quality and were redistilled before use. Absolute ether was passed through a column of Al_2O_3. Care was taken to use the purest solvents available, otherwise traces of unknown impurities tended to obscure parts of the spectrum. For this reason, a mixture of equal parts of pentane and hexane ('P.H.') (American Mineral Spirits Co., New York) was used as a solvent, with properties similar to those of light petroleum. Proportions of all solvent mixtures are v/v.

Chromatography. Columns of dry silica gel were used (Dobriner, personal communication). A column was prepared by filling a suitable tube with P.H. The appropriate quantity (usually 200 times the weight of the esters) of silica gel (grade 923, through 200, Davison Chemical Corporation, Baltimore) was then added in a fine stream and the solvent allowed to flow until a compact column was formed. The esters were applied as a solution in a solvent of relatively low polarity (e.g. P.H., 90:ether, 10), and development proceeded with solvent mixtures of increasing polarity. In a typical chromatograph, the solvent mixtures were: (1) P.H., 99:ether, 1 to dissolve the esters. (2) Mixtures of P.H. with ether containing 5, 10, 15, 20, 30, 40, 60 and 80% ether. (3) Pure ether. (4) Mixtures of ether and acetone, containing 5, 10 and 50% acetone. (5) Pure acetone. (6) Acetone, 50; methanol, 50. (7) Pure methanol.

Amounts of 100–250 ml. of each solvent mixture were applied, the eluate being collected in 50 ml. fractions at a rate of 2–3 drops/sec. If necessary, N_2 under pressure was admitted to the solvent reservoirs to maintain this rate of flow. Each fraction was taken to dryness at once and, if eluted material was present, the same solvent mixture was continued until no significant residue was found. The next solvent mixture was then applied.

Infrared spectrometry. The residues from all the individual fractions were dissolved in CS_2 and examined in the spectrometer. The instrument used was a Perkin-Elmer model 12, fitted with a sodium chloride prism and a 3 mm. cell of capacity 0·03 ml. (Colthup & Williams, 1947; Hardy, Wilson & Dobriner, 1949). With a cell of this capacity, about 100 μg. of ester were required to produce a well defined spectrum. Fractions containing a larger quantity were diluted appropriately.

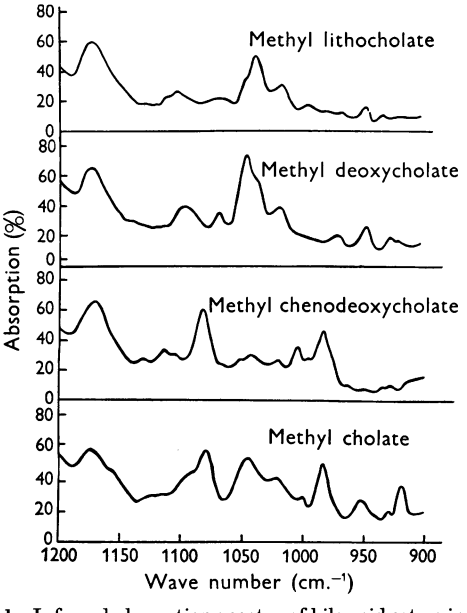

Fig. 1. Infrared absorption spectra of bile acid esters in CS_2 solution. The spectra cover the region of the molecular 'finger-print'.

The spectra were regularly recorded over the frequency range between 1180 and 875 cm.$^{-1}$. In this 'finger-print' region, all the many steroids so far examined show marked individuality (Jones & Dobriner, 1949), including the bile acids (Fig. 1). Whenever necessary, other regions of the spectrum were also recorded.

Melting points. A hot-stage microscope was used. All melting points are corrected.

Experiments with pure compounds

Experience in the separation procedure was gained by chromatographing mixtures containing the methyl esters of lithocholic acid (3-hydroxycholanic acid), deoxycholic acid (3:12-dihydroxycholanic acid), chenodeoxycholic acid (3:7-dihydroxycholanic acid) and cholic acid (3:7:12-trihydroxycholanic acid). It was found that these compounds fell into three well separated groups. Methyl lithocholate was eluted from the column first, followed by the two dihydroxy esters which were recovered as a mixture. Finally, pure methyl cholate was obtained.

Attempts were made to separate the dihydroxy esters by using a different solvent system (benzene and ethanol) and by chromatography on alumina. The best results, however, were obtained by acetylating the mixture of dihydroxy esters and rechromatographing the diacetates on silica gel. A fair separation was then possible, with methyl diacetyldeoxycholate being eluted first, but some overlapping usually occurred. The spectra of the binary mixtures were readily identified.

The approximate solvent mixtures required to elute different substances are given in Table 1, which also includes two additional compounds encountered later in this investigation. In different chromatograms, the various compounds always appeared in the same order and there were only slight variations in the mixtures required to elute a given substance.

Table 1. *Solvent mixtures required to elute compounds from silica gel*

Solvent mixture	Compound
Before acetylation	
P.H.*-ether 70:30	Cholesterol
P.H.-ether 40:60	Methyl lithocholate
P.H.-ether 20:80	Unidentified keto acid (not present in ox bile)
Ether	Methyl deoxycholate and methyl chenodeoxycholate
Ether-acetone 50:50	Methyl cholate
After acetylation	
P.H.-ether 85:15	Methyl diacetyldeoxycholate followed by methyl diacetylchenodeoxycholate

* P.H. is pentane-hexane (1:1).

Application to analysis of ox bile salts

Material. This was a preparation made by Abbott Laboratories, Chicago, containing the 90% ethanol-soluble constituents of fresh ox bile.

Hydrolysis. The pale yellow powder (4·1 g.) was dissolved in water (75 ml.) and mixed with a solution of 8 g. NaOH in 15 ml. water. The solution was heated on a steam bath for 20 hr., made acid with 10 N-HCl, saturated with solid NaCl and allowed to stand for some hours. The solid material was washed with water, dried and exhaustively extracted with boiling ethanol. The ethanolic solution yielded 2·5 g. of a brown gum containing the mixed bile acids.

Methylation. The crude acids were dissolved in methanol and treated at 0° with an excess of ethereal diazomethane solution: after evaporation of the solvent, the residue was dissolved in ether. The solution was washed with 0·2 N-NaOH, dried and taken to dryness, leaving the mixed esters as an amber gum weighing 2·3 g.

All subsequent work was done on a portion of the mixed esters weighing 250 mg.

Initial chromatogram. The material (250 mg.) was dissolved in 250 ml. P.H.-ether (99:1) and applied to a column 17 cm. high, 2·2 cm. diameter, containing 50 g. silica gel. Subsequent development produced 102 fractions of 50 ml. each, summarized in Table 2.

Fractions 42–50. The combined material was dissolved in P.H.-methanol and yielded a few crystals, m.p. 118–119° not depressed by mixture with authentic methyl lithocholate, m.p. 123°.

Fractions 80–92. This material crystallized as leaflets from P.H.-ether, m.p. 151–2°, raised by recrystallization

from P.H.-ethanol to 158° unaltered by mixture with methyl cholate, m.p. 157–158°.

Second chromatogram. The material in fractions 56–70 was acetylated at room temperature with 0·1 ml. acetic anhydride and 0·1 ml. pyridine. The product was dissolved in 100 ml. P.H.-ether (95:5) and chromatographed on a column 28 cm. high, 0·7 diameter, containing 8 g. silica gel. Development was carried out with P.H.-ether (85:15), the eluate being collected in 50 ml. fractions. Pure methyl diacetyldeoxycholate was found by infrared spectrometry in fractions 9–22. The combined material crystallized from aqueous ethanol as long needles, m.p. 116–117°.

Fractions 23–30 were combined and weighed 1·5 mg. From its spectrum it was evident that this material consisted of a mixture of methyl diacetyldeoxycholate and methyl diacetylchenodeoxycholate. Accordingly, mixtures containing various proportions of these two esters were prepared and submitted to spectrometry. The spectrum of 60% methyl diacetyldeoxycholate and 40% methyl diacetylchenodeoxycholate matched most closely the spectrum of the combined fractions (Fig. 2). It was concluded that the latter contained 0·6 mg. methyl diacetylchenodeoxycholate and 0·9 mg. methyl diacetyldeoxycholate.

Table 2. *Initial chromatogram of mixed esters from ox bile*

(Fractions were 50 ml. each. All fractions not described contained negligible amounts of material.)

Fractions	Eluant	Description	Identification by infrared spectrometry, etc.
8–11	P.H.-ether 95:5	Trace of white solid	Unknown, negative Pettenkofer reaction
26–31	P.H.-ether 70:30	1·1 mg. waxy solid	Cholesterol
42–50	P.H.-ether 40:60	1·8 mg. colourless gum	Methyl lithocholate
56–70	Ether 100	38·6 mg. colourless gum	Methyl deoxycholate
80–92	Ether 50; acetone 50 and onwards	181 mg. yellow gum	Methyl cholate

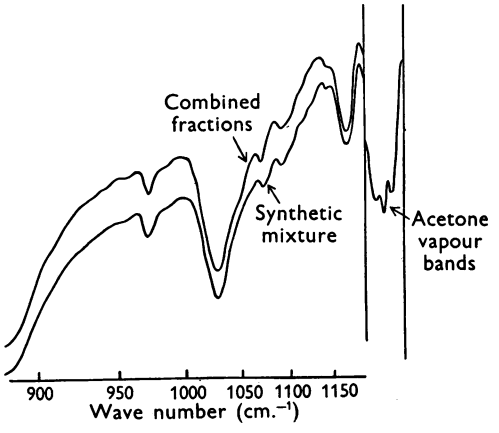

Fig. 2. The infrared spectrum of material isolated from ox bile salts compared with that of a mixture of 60% methyl 3:12-diacetoxycholanate and 40% methyl 3:7-diacetoxycholanate. The spectra are traced from the original record and appear against a sloping background of changing energy. Absorption bands are registered as downward-pointing 'peaks' and a wavelength marker of acetone vapour bands is included.

DISCUSSION

Chromatography has often been found useful for separating mixtures of steroids (for references see Reichstein & Shoppee, 1947). As a method of identifying the separated bile acid esters, infrared spectrometry possesses considerable advantages. Experience with the spectra of steroids has shown that their specificity is beyond question (Jones & Dobriner, 1949). In the work described here, crystalline esters were obtained when possible, but the primary identification was made by examining the spectra, and the mixed melting points were regarded as only confirmatory. Thus a compound like chenodeoxycholic acid, whose derivatives are difficult to crystallize, can be identified as easily as cholic acid.

The amount of material required is well illustrated by the results of the second chromatogram, in which less than 1 mg. of methyl diacetylchenodeoxycholate was found, from the total 250 mg. of mixed esters. No bile acids were encountered other than the four known to be present in ox bile. The keto acids described by Wieland & Kishi (1933) and Haslewood (1946) were not encountered; in view of the minute amounts which these authors isolated, this result was not surprising.

When analysing urinary steroids, Dobriner *et al.* (1948) made use of specific reactions (e.g. the Zimmermann-Callow reaction) to determine the amount of steroid material present in their various fractions. In the absence of such specific reactions, it has been necessary in this work to weigh the fractions as an estimate of the amount of steroid present. The esters are obtained in a fairly pure form, as indicated by their spectra and by the ease with which they crystallize, so that it is considered that the weights form a reasonably accurate estimate. However, it is hoped to develop alternative methods, one possibility being to make absorption measurements of the band at 1742 cm.$^{-1}$ characteristic of the —$COOCH_3$ group.

SUMMARY

1. Chromatography on silica gel, combined with infrared spectrometry, has been used to analyse mixtures of bile acids.

2. The results obtained by applying this method to an ox bile-salt preparation are described. Methyl lithocholate (methyl 3-hydroxycholanate) (1·8 mg.), methyl deoxycholate (methyl 3:12-dihydroxycholanate) (38 mg.), methyl chenodeoxycholate (methyl 3:7-dihydroxycholanate) (0·6 mg.) and methyl cholate (methyl 3:7:12-trihydroxycholanate) (181 mg.) were recovered from 250 mg. of crude mixed esters.

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Enzyme inhibitions by Snake Venoms

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Snake venoms are complex mixtures of components, many of which are known to be proteins having enzymic activities. Their enormous toxic power is illustrated by the fact that the minimum lethal dose for mice of a purified preparation of neurotoxin from cobra venom is 9·0–0·03 μg./g. weight of animal (Micheel & Jung, 1936). The complex nature of venoms gives rise to a variety of symptoms whose dominant clinical features have caused the venoms to be classified as neurotoxic, haemotoxic or depressant.

Although the presence of proteolytic enzymes in venoms was described by de Lacerda (1881), it was only after an investigation of the coagulating effect of some varieties of venoms (viperine) (Lamb, 1901; Noc, 1904; Martin, 1905) had stimulated the study of venom proteases, and after Delezenne & Ledebt (1911*a*, *b*) had correlated the presence of lecithinase with haemolysis, that it became evident that enzymes may play a significant role in the pathogenic effects of venoms. Feldberg & Kellaway (1937, 1938) have pointed out that the effects of cobra venom on the circulatory system may be accounted for, to some extent, by the presence of a phosphatidase.

A considerable variety of enzymes is now known to exist in snake venoms. These are proteolytic enzymes, phosphatidases, hyaluronidase, amino-acid oxidase, phosphoesterases, 5-nucleotidase, ribonuclease and deoxyribonuclease, cholinesterase, adenosinetriphosphatase and diphosphopyridine nucleotidase. (For review see Zeller, 1951.) How far these enzymes, or other components of venoms, are implicated in the production of neurological lesions by venoms is still quite obscure.

The variety of symptoms produced by venoms on the nervous system has been attributed to components loosely referred to as 'neurotoxins'. Most investigations have pointed to the protein nature of neurotoxins. A long time ago Mitchell (1860) separated a protein fraction from rattlesnake venom which was toxic to pigeons. Slotta & Fraenkel-Conrat (1938) reported the isolation of a neurotoxin of *Crotalus terrificus terrificus* in crystalline form which they termed 'crotoxin'. This protein has been found to possess phosphatidase activity and to