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 infrared spectrometry (Dobriner, Lieberman & Rhoads, 1948; Dobriner et al. 1948b; 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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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 $\mu$g of ester were required to produce a well defined spectrum. Fractions containing a larger quantity were diluted appropriately.

![Infrared absorption spectra of bile acid esters in CS$_2$ solution.](image)

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 diacetics on silica gel. A fair separation was then possible, with methyl diacetyl-deoxycholate 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>
<thead>
<tr>
<th>Solvent mixture</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.H.*-ether 70:30</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>P.H.-ether 40:60</td>
<td>Methyl lithocholate</td>
</tr>
<tr>
<td>P.H.-ether 20:80</td>
<td>Unidentified keto acid (not present in ox bile)</td>
</tr>
<tr>
<td>Ether</td>
<td>Methyl deoxycholate and methyl chenodeoxycholate</td>
</tr>
<tr>
<td>Ether-acetone 50:50</td>
<td>Methyl cholate</td>
</tr>
<tr>
<td>P.H.-ether 85:15</td>
<td>Methyl diacetyldiheoxycholate followed by methyl diacetyl-chenodeoxycholate</td>
</tr>
<tr>
<td>* P.H. is pentane-hexane (1:1).</td>
<td></td>
</tr>
</tbody>
</table>

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 diacetylenodeoxycholate. Accordingly, mixtures containing various proportions of these two esters were submitted to spectrometry. The spectrum of 60% methyl diacetyldeoxycholate and 40% methyl diacetylenodeoxycholate 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 diacetylenodeoxycholate.

**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 diacetylenodeoxycholate 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.

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

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

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

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.
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 3:7:12-trihydroxycholanate) (0.6 mg.) and methyl cholate (methyl 3:7:12-trihydroxycholanate) (181 mg.) were recovered from 250 mg. of crude mixed esters.

My grateful thanks are due to Dr C. P. Rhoads for enabling me to work in the Sloan-Kettering Institute for Cancer Research. Dr Konrad Dobriner and Dr R. Norman Jones gave constant help and encouragement, while Dr T. F. Gallagher was generous in providing pure reference compounds. I am also indebted to many other members of the staff of the Sloan-Kettering Institute for their unfailing courtesy and patience.

This work was done during the tenure of an American Government grant under the Fulbright and Smith-Mundt Acts. Additional funds were kindly made available by the Trustees of the Sloan-Kettering Institute.

REFERENCES


Enzyme inhibitions by Snake Venoms

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(Received 14 March 1952)

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 0.0 - 0.03 µg./g. weight of animal (Michel & Jung, 1938). 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 Delezene & Ledebt (1911a, 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 diphasphopyridine 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 'crototoxin'. This protein has been found to possess phosphatidase activity and to