Comparative Studies of 'Bile Salts'

8. PRELIMINARY EXAMINATION OF BILE SALTS BY PAPER CHROMATOGRAPHY

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Methods for the examination of free bile acids have been greatly improved in recent times and progress in this respect can be said to be satisfactory (see, for example, Bergström & Sjövall, 1951; Sjövall, 1953; Wootton, 1953).

However, until very recently no real advance was made in methods for the study of the native bile salts themselves. Ahrens & Craig (1952) showed that counter-current distribution could be applied with success to ox bile salts, and they published the first comprehensive analysis of this material. Subsequently, Sjövall (1952; 1954) demonstrated that paper-partition chromatography could be used for the same purpose. Bergström & Norman (1953*a*) and Norman (1953) used partition chromatography for the separation of bile acids as they occur in bile and faeces. Bile acids conjugated with taurine could be separated as a group, and free bile acids or those conjugated with glycine could be separated as individual compounds by the use of different solvent systems.

The present report is of work designed to investigate the application of the paper chromatography of Sjövall (1952, 1954) to biological problems.

It was decided to investigate especially (*a*) the occurrence of bile acids conjugated with glycine in vertebrates other than mammals and (*b*) species known or thought to have remarkable bile salts.

Systematic names for free bile acids are as follows: cholic acid (3α:7α:12α-trihydroxycholanic acid); pythocholic acid (probably 3α:12α:16α-trihydroxycholanic acid); deoxycholic acid (3α:12α-dihydroxycholanic acid); chenodeoxycholic acid (3α:7α-dihydroxycholanic acid); hyodeoxycholic acid (3α:6α-dihydroxycholanic acid); ursodeoxycholic acid (3α:7β-dihydroxycholanic acid); trihydroxycoprostanic acid (3α:7α:12α-trihydroxycoprostanic acid). Glycine and taurine conjugates are indicated by the prefixes 'glyco' and 'tauro'.

METHODS

Paper chromatography (see Sjövall, 1952, 1954). This was carried out using systems as follows: Glyco system, G_1: mobile phase, 75% (v/v) *iso*propyl ether/*n*-heptane; stationary phase, 70% (v/v) acetic acid/water. Glyco system, G_2: mobile phase, 85% (v/v) *iso*propyl ether/*n*-heptane; stationary phase, as G_1. Glyco system, G_3: mobile phase, 60% (v/v) *iso*propyl ether/*n*-heptane; stationary phase, as G_1. Tauro system, T: mobile phase, *n*-butanol equilibrated with 3% (v/v) acetic acid/water; stationary phase, 70% (v/v) acetic acid/water.

The mobile and stationary phases of G_1, G_2 and G_3 were equilibrated by shaking and separating at room temperature; such equilibration cannot be carried out with system T. Whatman's no. 3MM paper was prepared for all systems by washing with 70% (v/v) acetic acid/water and heating until superficially 'dry' in an oven (about 5 min. at 90°). The spots of bile salt solutions were then applied at the starting line (about 10 cm. from one end of the paper) in drops, the approximate volume (0·008 ml.) of which had been previously determined by weighing. Hence, it was possible to calculate the figures, given in Table 1, for μg. of bile salt put on to the starting line.

Chromatography (ascending) with glyco systems G_1 and G_2. A strip of paper (about 12 × 40 cm.) treated and loaded with bile salts as above was hung (with the starting line at the bottom) in a glass tank tilted so that mobile phase on the floor of the tank was out of contact with the paper. For equilibration, wide strips of Whatman's no. 1 paper moistened with and dipping into the mobile phase were placed in the tank around the walls, and other paper strips were dipped into and left saturated with stationary phase, contained in a beaker also in the tank. The tank was sealed first with a cloth wetted with mobile phase, then a cellophan cover and finally with a rubber cover. After about 12–16 hr. at about 20–23°, when equilibration was presumed to be complete, the tank was tilted so that the mobile phase ran on to the bottom of the paper. Ascension of the solvent front to about 20 cm. above the starting line took 2–4 hr. and the paper was then removed and dried at 90–100°. It was sprayed with 10% (w/v) phosphomolybdic acid in ethanol (Kritchevsky & Kirk, 1952) and heated at about 100° until the blue spots developed (about 5 min.).

Chromatography (descending) with tauro system T and glyco system G_3. The paper, treated and loaded with bile salts as described above, was hung (with the starting line at the top) from a trough in a glass tank so that the starting line was about 10 cm. from the trough and the bottom of the paper clear of the floor of the tank. Mobile phase covered the floor of the tank and also saturated strips of Whatman's no. 1 paper on the walls. Stationary phase in a beaker in the tank ran up a paper strip. The tank was sealed with a cloth, cellophan and then a rubber cap. After about 16 hr., these were pierced with a hypodermic syringe and mobile phase was thus injected into the trough. The liquid was allowed to run down the paper for a total distance of about 30 cm.

When G_3 was used, the chromatogram was run for 18 hr., the mobile phase dripping off the lower edge of the paper. The paper was dried and sprayed as described above.

Standard solutions. Determination of R_F values was of little use in the present work; the values varied considerably with temperature and equilibration conditions, and this is perhaps not surprising in view of the method used to establish a stationary phase in the paper (see above). However, reliable and consistent results were obtained by comparison with standard bile salt preparations run on the same paper. Sodium salts of taurocholic, taurodeoxycholic, glycocholic and glycodeoxycholic aicds were prepared as described by Cortese (1937) and purified by passage through the ion-exchange resin Amberlite IRA-400 (OH) (Rohm and Haas Co., Philadelphia, U.S.A.), which removed inorganic impurities. Glyco- and tauro-chenodeoxycholic acids were prepared as described by Bergström & Norman (1953*b*). Examination of the chromatograms suggested that the cholic acid derivatives might have contained a little free cholic acid. For the present work 'synthetic' conjugated hyodeoxycholic acids were not available, and the situation with respect to the behaviour on paper of these substances is therefore not quite clear (see Discussion). Taurine conjugates of deoxycholic and of chenodeoxycholic acids ran at the same rate with system *T*. With systems G_1 and G_2, glycodeoxycholic and glycochenodeoxycholic acids ran at closely similar rates.

Standard solutions contained 2–5 mg./ml. in water (cholic acid in ethanol). With the spraying reagent used it was possible to detect about 0·8 μg. of glycocholate and 1·6 μg. of taurocholate, and these figures were used as the basis for the calculation of columns 3 and 4 in Table 1.

Preparation of bile salts. It was found, after preliminary trials, that crude bile salts as prepared by Haslewood & Wootton (1950) could be directly dissolved in water and applied to the papers. This procedure resulted in some streakiness, due probably to impurities in the bile salts, but this did not impair the visibility of the spots. The use of crude bile salts greatly shortened the time needed for analysis.

RESULTS

Examination for glycine conjugation. Examples of papers obtained were photographed and are shown in Figs 1 and 2. Table 1 indicates that in the bile salts of seven species of fish studied, glycocholic acid could not have constituted more than about 0·7 % and glycodeoxycholic acid not more than about 1·3 %. The corresponding figures for eight species of birds were 0·8 and 1·6 %. Glycine conjugates were not detected in the chicken, dogfish or frog (Fig. 2) and probably not in the wallaby (Fig. 1); they could be found in the goat and whale (Fig. 1).

The significance of these findings is more fully discussed below.

Examination of various bile salts. These can be divided into groups, as shown in Table 2, according to whether they are known or expected to contain

Table 1. *Examination of the bile salts of fish and birds for glycocholic or glycodeoxycholic acids by paper chromatography*

Details of the method are described in text.

Species	Approx. concentration of bile salts (mg./ml.)	Approx. wt. put on start line (μg.)	Minimum amount of glycocholic acid detectable (%)	Minimum amount of a glycodeoxycholic acid detectable (%)	Notes on bile
Cod, *Gadus morrhus*	28·7	229	0·3	0·7	Contains cholic acid (Haslewood & Wootton, 1950)
Plaice, *Pleuronectes platessa*	17·1	137	0·6	1·2	As above
Flounder, *P. flesus*	16·1	129	0·6	1·2	
Bass, *Morone labrax*	17·8	142	0·6	1·1	Expected to contain taurocholate
Brown trout, *Salmo trutta*	14·9	119	0·7	1·3	
Rainbow trout, *S. irideus*	19·9	159	0·5	1·0	
Dogfish, *Acanthias vulgaris*	19·4	155	0·5	1·0	Contains scymnol, or a precursor, conjugated with sulphate (Cook, 1941)
Domestic chicken	17·9	143	0·6	1·1	
Golden eagle, *Aquila chrysäetus*	21·2	169	0·5	0·9	
Bateleur eagle, *Terathopius ecaudatus*	21·5	172	0·5	0·9	
Eagle Owl, *Bubo bubo*	17·8	142	0·6	1·1	
Shoebill, *Balaeniceps rex*	12·8	102	0·8	1·6	See Discussion
Cassowary, *Cassuarius rogersi*	15·0	120	0·7	1·3	
King penguin, *Aptenodites patogonica*	21·0	168	0·5	0·9	
Black-thighed Hornbill, *Bycanistes cylindricus*	17·3	138	0·6	1·2	

Table 2. *Results of examination by paper chromatography of bile salts of various species*

References to Table 1 are to Latin names, concentrations and notes on bile. Abbreviations: *M*, modern; *P*, primitive; *MP*, most primitive; G_3, *T*, glycine, taurine systems, respectively; *TC*, spot as taurocholate; *TD*, spot as taurodeoxycholate.

Species (The figures in parentheses refer to the approx. concentration of bile salts in mg./ml. used for the experiment)	Notes on bile salts	System	References to figures	Results*
Pig (15·3); Coypu, *Myocastor coypus* (11·3)	*M*, see Discussion	G_3 & *T*	3	*TD* (on starting line in Fig. 3) and see Discussion
Dog, *Canis familiaris* (19·4)	*M*	*T*	—	*TC* and *TD*
Wallaby, *Macropus ruficollis* (20·8); domestic goat (21·0); fin whale, *Balaenoptera physalus* (17·4); polar bear, *Thalarctos maritimus* (18·4)	All *M*	*T*	—	*TC* and *TD*
Himalayan bear, *Ursus tibetanus* (21·3)	*M*, possibly contains ursodeoxycholate	*T*	—	*TD* and an unidentified spot
Cassowary (Table 1)	See Discussion	*T*	—	Entirely anomalous; see Discussion
King penguin and eagle owl (Table 1)	*M* and *P*, see Discussion	*T*	—	*TC* and *TD*
Diamond rattlesnake, *Crotalus adamanteus* (20·6)	*M*. Almost certainly taurocholate	*T*	—	*TC*, possibly *TD*. Two or three unidentified spots
Python reticulatus (15·0)	*M*. Pythocholic and cholic acids	*T*	—	Spot slightly in advance of *TC*. ? Unidentified spot
Carpet python, *P. spilotus* (20·0)	*M*. Probably pythocholic acid	*T*	—	Spot in advance of *TC*. Probably *TD*
Australian crocodile, *Crocodylus johnsonii* (19·3)	Probably *P*	*T*	4	As cayman (see below)
Cayman, *Caiman crocodilus* (16·2)	*P*. Contains taurotrihydroxycoprostanate (Haslewood, 1952*a* and unpublished results)	*T*	4	Spots running faster than *TC* and at about the same rate as *TD*
Green turtle, *Chelone midas* (20·2)	Possibly *P*. Contains unidentified bile acids (Haslewood & Wootton, 1950)	*T*	4	Two spots, running close to but more closely than *TC* and *TD*
Frog, *Rana temporaria* (19·5)	*MP*. Mainly ranol sulphate (Haslewood, 1952*b*)	*T*	5	Almost as *TC*. ? *TD*
Gaboon viper, *Bitis gabonica* (20·4)	Unidentified bile salts (Haslewood & Wootton, 1950)	*T*	5	Two spots running more slowly than but close to *TC* and *TD*
Dogfish (Table 1)	*MP*. See Table 1	*T*	5	*TC*. ? *TD*
Mirror carp, *cyprinus carpio* (20·8)	*MP*. (Haslewood, 1951)	*T*	6	Spots differ from *TC* or *TD*
Tench, *Tinca vulgaris* (19·1)	Unidentified; the tench is a close relative of the carp	*T*	6	As carp (above)
Cod, flounder, plaice, bass (see Table 1)	All certainly or probably *M*	*T*	6	*TC* and *TD*

* Spots corresponding to taurocholate or taurodeoxycholate cannot be taken as necessarily indicating the presence of these substances (see text).

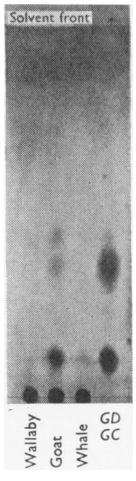

Fig. 1.

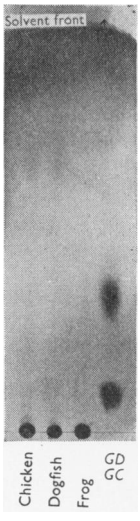

Fig. 2.

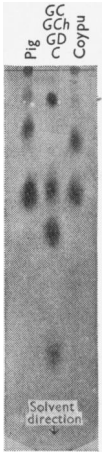

Fig. 3.

Figs. 1 and 2. Paper chromatograms of bile salts of species named. Ascending chromatography as described in Methods. Fig. 1: glyco system G_1. Fig. 2: glyco system G_2. *GD*, glycodeoxycholate standard (upper spot); *GC*, glycocholate standard (lower spot). Taurine conjugates remain at starting line. For interpretations, see Results and Table 2.

Fig. 3. Paper chromatograms of bile salts of pig and coypu. Descending chromatography as described in Methods, with use of glyco system G_2. Standards (from bottom of photograph upwards): *C*, cholic acid; *GD*, glycodeoxycholate; *GCh*, glycochenodeoxycholate; *GC*, glycocholate. For interpretations, see Table 2 and Discussion.

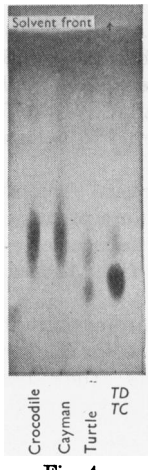

Fig. 4.

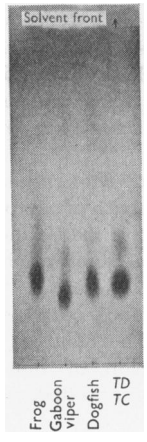

Fig. 5.

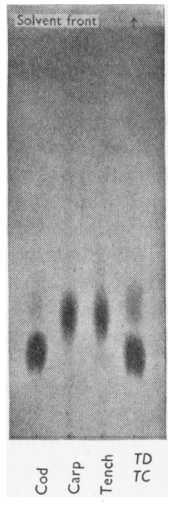

Fig. 6.

Figs. 4–6. Paper chromatograms of bile salts of species named. Descending chromatography as described in Methods, with use of tauro system *T*. *TD*, taurodeoxycholate standard (upper spot on photographs); *TC*, taurocholate standard (lower spot on photographs). For interpretations, see Table 2.

(i) C_{27} or C_{28} alcohols conjugated with sulphate ('most primitive'), (ii) C_{27} or C_{28} acids conjugated with taurine ('primitive'), or (iii) C_{24} acids conjugated with taurine or glycine ('modern').

Results are summarized in Table 2 and photographs of chromatograms are shown in Figs. 3–6.

DISCUSSION

Our failure to find glycine conjugates in the birds and fish examined tends to strengthen the working hypothesis of Haslewood & Wootton (1950) that 'glyco acids are apparently found only in mammalia'. However, it should be pointed out that in mammals bile salts containing a very high proportion of such conjugates seem to be found only in vegetarian species. For the present study, none of the few vegetarian reptiles and no herbivorous fish containing known bile salts were available; examination of such animals for glycine conjugates ought to be undertaken. Arnstein & Neuberger (1953) have suggested that in birds glycine is a 'semi-essential' amino acid, and amounts sufficient for the synthesis of glyco bile salts might not be expected to be readily available.

The results summarized in Table 2 call for certain detailed comments.

(*a*) The pig and coypu chromatograms (Fig. 3) each show spots corresponding to glycochenodeoxycholic acid, but not to glycodeoxycholic acid. Chenodeoxycholic acid has in fact been isolated from the bile of both animals (Ido & Sakurai, 1939; Haslewood, 1954). In the coypu, a spot corresponds to glycocholic acid; cholic acid has been found in the bile (Haslewood, 1954). In the pig, the 'glycocholic' spot ran at a somewhat slower rate than authentic glycocholic acid, suggesting that it might be due to a different substance; the phenomenon is certainly worth further investigation. The remaining spot in the pig chromatogram may be due to glycohyodeoxycholic acid and that in the coypu to glycoursodeoxycholic acid (Kazuno, 1946; Haslewood, 1954).

(*b*) It is evident from Fig. 3 and from unpublished chromatograms that pig and coypu bile salts contain small amounts of substances resembling taurodeoxycholate; tauro conjugation is probably not entirely suppressed in these species.

(*c*) The bile salts of the birds mentioned in this report are at present being examined by Dr I. G. Anderson in these laboratories, and the assessment of king penguin bile as showing both 'modern' and

'primitive' characteristics is due to him. Yamasaki (1951) has isolated a bile acid (probably C_{27}) from chicken bile salts, the chief constituent of which is, of course, taurochenodeoxycholate. Dr Anderson has found that our specimen of the 'bile salts' of the cassowary is indeed anomalous in its behaviour; he has suggested that it may consist largely of the fat and other materials normally excreted in variable amounts in bile.

(*d*) It is interesting that ranol sulphate (frog bile, Fig. 5) and the sulphate of scymnol (or a precursor) (dogfish, Fig. 5) both run at almost the same rate as taurocholate; the alcohol sulphate from the carp (Fig. 6) is not very different.

The simple methods of paper chromatography now tested should be of great value for the preliminary examination of bile salts; they have already indicated several lines along which progress may be made.

SUMMARY

1. Simple methods of paper chromatography developed by Sjövall (1952, 1954) have been applied to a search for bile acids conjugated with glycine in the bile salts of seven species of fish and eight species of birds and to the examination of the crude bile salts of a number of vertebrates.

2. No glycine conjugates were found in the fish and birds examined.

3. Some implications of the results have been discussed.

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The Phosphatides of the Latex of *Hevea brasiliensis*

2. PURIFICATION AND ANALYSIS

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Some properties of latex phosphatide were reported in a previous paper (Smith, 1954), but it was found that an extensive analysis could not be usefully carried out without first removing water-soluble impurities which were carried into solution in lipid solvents by the phosphatides. Workers with a variety of phosphatides from both animals and plants have encountered similar difficulties and have used a number of methods for purification. Thus Channon & Foster (1934), suspended wheat-germ phosphatide in water, and flocculated it by adding acetone and sodium chloride. This method was used by Tristram (1942) for the purification of latex phosphatide. Folch & Van Slyke (1939) and Christensen (1939) washed solutions of blood phosphatides in light petroleum with water. McKibbin & Taylor (1949) found that the large losses of phosphatide incurred by using this method could