3. Evidence for the biosynthesis of condensed tannins from monomeric leuco-anthocyanins is presented.

4. Conversions persist into the central heartwood, suggesting that these cells still contain active-enzyme systems at an advanced age, even after heavy deposition of tannin.

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Studies in the Bile Acids

3. THE CONJUGATED BILE SALTS OF CERTAIN PRIMATES*

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In a previous paper, the non-ketonic acids of human bile were described (Wootton & Wiggins, 1953). The major constituent was shown to be chenodeoxycholic acid (3 α :7 α -dihydroxycholanolic acid) and it was suggested that the presence of a high proportion of this acid might be characteristic of the human species. To test this suggestion, a series of primate biles have now been examined.

The human-bile analyses reported in 1953 were done by hydrolysing the conjugated bile salts and submitting the bile acids produced (after methylation) to chromatographic separation and identification by infrared spectroscopy. No distinction was therefore made between glycine-conjugated and

taurine-conjugated acids. However, Ahrens & Craig (1952) have developed a countercurrent method capable of separating these two classes of conjugates and this procedure has been applied as the initial stage of the primate-bile analyses. The separated fractions of the bile have then been carried separately through the remainder of the analysis. This complete method has been applied to samples of gall-bladder bile derived from the human (three specimens), the chimpanzee (*Pan satyrus*), the white-nosed monkey (two specimens of *Cercopithecus nictitans mortinii*), the mona monkey (*Cercopithecus mona*) and the tantalus guernon (*Cercopithecus aethiops tantalus*). The specimens of monkey bile were kindly provided by Professor G. A. D. Haslewood.

* Part 2: Wootton & Wiggins (1953).

EXPERIMENTAL

Material. Bile from all species was obtained at autopsy within a few hours of death. The contents of the gall bladder were mixed with 2 vol. of ethanol, allowed to stand for 24 hr., filtered and evaporated to dryness.

Countercurrent separation. The apparatus used was an all-glass system with a maximum capacity of thirty tubes. Each tube required 40 ml. of upper phase and 40 ml. of lower phase. When a transfer was done, the upper phases moved while the lower phases remained in the same tubes. The separation was done in two stages. The first stage removed the cholesterol and lipid material, leaving purified bile salts. The solvent system consisted of 97.5% (v/v) acetic acid–heptane (Ahrens & Craig, 1952), shaken together and allowed to separate. Six tubes were filled with lower phase, and the ethanol-soluble bile fraction (about 1 g.) was dissolved in the first tube. Fifteen upper phases were then passed through the apparatus. The contents of the first four tubes were combined and evaporated to dryness, yielding purified bile salts.

The second stage separated the glycine conjugates from the taurine conjugates. The solvent system was 10% (v/v) acetic acid (lower phase) equilibrated with an equal volume of heptane–butanol (70:30, v/v) (upper phase). Nine tubes were filled with lower phase and the purified bile salts were dissolved in the first tube. Twenty-five upper phases were then passed through and transfers continued until all the upper phases had left the tubes. The first 22 upper phases, containing the glycine-conjugated fraction, were combined and evaporated to dryness. The contents of the first six tubes were combined and dried to give the taurine-conjugated fraction.

Test of countercurrent separation. It was considered advisable to test both stages of the separation. This was done for the initial purification by submitting a bile specimen to an extended experiment with a larger number of tubes. A quantity (0.9 g.) of the ethanol-soluble material from bile sample 2 (Table 1) was distributed between 30 tubes by using the acetic acid–heptane system. The upper and lower phases of each tube were separately evaporated to dryness and tested for the presence of cholesterol by the Liebermann–Burchard reaction (King, 1951) and cholic acid (3 α :7 α :12 α -trihydroxycholic acid) by a modified Pettenkofer reaction (see below). Positive results are shown in Table 1.

The second stage of the separation was tested similarly by distributing 1.07 g. of purified bile salts from sample 1 between 30 tubes by using the acetic acid–heptane–butanol system. The bile salts were dissolved in the lower phase of the first tube and 33 upper phases were passed successively into the system. The three upper phases which passed through the last tube were collected together. The contents of the various tubes were separately evaporated to dryness and a portion of the material in each tube was tested for cholic acid by the modified Pettenkofer reaction. Another measured portion (1 mg.) was dissolved in 5 ml. of 6*N*-hydrochloric acid, heated at 100° for 24 hr. and evaporated to dryness in a current of air. Water (2 ml.) was then added and evaporated; this procedure was repeated twice more to remove traces of hydrochloric acid. The hydrolysed residue was dissolved in a few drops of water and examined for amino acids by ascending paper chromatography, with a collidine–lutidine–water solvent and ninhydrin spray.

Markers of glycine and taurine were run with each chromatogram.

In preliminary experiments, as many as six ninhydrin-reacting spots were seen. To remove free amino acids present in the purified bile salts, the hydrochloric acid used in the hydrolyses was therefore shaken with solid silver nitrite. Results obtained after this procedure are shown in Table 2. There is a marked discontinuity in the distribution of Pettenkofer-reacting material, and the taurine conjugates are confined to the first three tubes. However, glycine, which must have been present as conjugates in the purified bile salts, appears in all tubes. This is not surprising since many substances are conjugated with glycine.

Table 1. *Test of first stage of countercurrent separation*

Bile sample 2; 0.9 g. of ethanol-soluble material. Solvent system: 97.5% (v/v) acetic acid–heptane. The figures in the fourth column are the approximate weights derived from the individual fractions before combination and final drying.

Lower phase no.	Liebermann–Burchard reaction	Pettenkofer reaction	Wt. (mg.)
1	Negative	Positive	800
2	Negative	Positive	
3	Negative	Faintly positive	
4–8 (combined)	Negative	Negative	7
Upper phase no.			
15–12 (combined)	Negative	Negative	27
11–9 (combined)	Faintly positive	Negative	15
8–1 (combined)	Positive	Negative	245

Table 2. *Test of second stage of countercurrent separation*

Bile sample 1; 1.07 g. of purified bile salts. Solvent system 10% (v/v) acetic acid/heptane–butanol (70:30, v/v).

Tube no.	Pettenkofer reaction	Amino acids detected	Wt. (mg.)
1	Positive	Glycine + taurine	531
2	Positive	Glycine + taurine	76
3	Faintly positive	Glycine + taurine	30
4	Negative	Glycine	20
5–13 (combined)	Negative	Glycine	54
14	Faintly positive	Glycine	2
15–30 (combined)	Positive	Glycine	139
Upper phases 1–3	Positive	Glycine	167

From the results of these tests, the abbreviated procedure described above was devised. Final tests with mixtures of pure synthetic glyco- and tauro-cholic acid showed that separation was complete and recoveries of over 90% were obtained.

Further stages in analysis. The glycine- and taurine-conjugated fractions were separately hydrolysed, methylated, chromatographed on silica-gel columns and examined in the infrared spectrophotometer as described by Wootton & Wiggins (1953).

Modified Pettenkofer reaction. The reagent was prepared by cooling an aqueous solution of fructose (1 mg./ml.) in ice-water and adding dropwise an equal volume of concentrated sulphuric acid with constant mixing and cooling. The reagent remained colourless for several hours at 0°. A solution of the material to be tested (1 mg.) in 2N-NaOH (2 ml.) was mixed with 2 ml. of reagent, heated for 2 min. in a boiling-water bath and cooled immediately. A simultaneous reagent blank was carried through and another similar portion of the material was treated with aq. 50% (v/v) H₂SO₄ in place of the reagent.

The reagent blank was clear-yellow in colour, while the sulphuric acid tube usually showed slight charring. If the material contained cholic acid or its conjugates, the test solution had a cerise-red colour. When conducted in this way, the test was negative with lithocholic acid (3 α -hydroxycholic acid), deoxycholic acid (3 α :12 α -dihydroxycholic acid) and chenodeoxycholic acid.

RESULTS

The complete analyses of the eight bile samples are presented in Table 3. The results are expressed as percentages calculated from the isolated methyl esters. In all species, the major part of the bile acids was found to be a mixture of cholic acid, chenodeoxycholic acid and deoxycholic acid, but

minor components were also occasionally detected. Lithocholic acid was present in the bile of *Cercopithecus mona* and a keto acid, 3 α :12 α -dihydroxy-7-oxocholanic acid, was found in *C. aethiops tantalus* and *C. nictitans mortinii*.

DISCUSSION

When the glycine- and taurine-conjugated fractions of a single bile sample are compared, the proportions of the major constituent acids are the same within experimental error. An exception is specimen 7, which was available only in very small amount; the results are therefore subject to a large experimental error. The proportion of taurine conjugates varies from 10 to 43% of the whole. Rosenthal & Falkenhausen (1923), as a result of the study of normal and pathological biles, postulated that the proportion of each conjugate present depends on the availability of taurine, which is used preferentially. The present results do not conflict with this view.

The position is probably the same with regard to the minor constituents, lithocholic acid and 3 α :12 α -dihydroxy-7-oxocholanic acid. These acids were detected only in the glycine fractions; if they were present in the same proportion in the taurine fractions, the absolute amount would have been below the lower limit of detection of the method. The quantity of material required to produce a spectrum which can be identified depends somewhat on the purity of the sample and in particular on the completeness with which it has been separated from other bile acids. In favourable circumstances rather less than 1 mg. is needed.

Table 3. Complete analysis of bile salts of eight primate biles

Sample	Species	Wt. of purified salts (g.)	Conjugate fraction (% of total salts)	Constituent acids (as percentage of glycine- or taurine-conjugated fraction)				
				Lithocholic acid	Cheno-deoxycholic acid	Deoxycholic acid	Cholic acid	3 α :12 α -Di-hydroxy-7-oxocholanic acid
1	Man	1.07	Glycine 67	—	43	29	28	—
			Taurine 33	—	44	30	26	—
2	Man	0.80	Glycine 57	—	46	20	34	—
			Taurine 43	—	49	21	30	—
3	Man	0.68	Glycine 90	—	44	19	37	—
			Taurine 10	—	42	18	40	—
4	<i>Pan satyrus</i>	0.28	Glycine 60	—	21	14	65	—
			Taurine 40	—	16	11	73	—
5	<i>Cercopithecus mona</i>	1.53	Glycine 87	3	5	40	52	—
			Taurine 13	—	4	39	57	—
6	<i>C. aethiops tantalus</i>	0.44	Glycine 85	—	13	51	32	4
			Taurine 15	—	13	51	36	—
7	<i>C. nictitans mortinii</i>	0.15	Glycine 46	—	13	20	66	—
			Taurine 54	—	22	33	45	—
8	<i>C. nictitans mortinii</i>	0.42	Glycine 92	—	5	39	53	3
			Taurine 8	—	4	40	56	—

If the results presented here are combined with those previously reported for human-bile constituents (Wootton & Wiggins, 1953), it will be seen that, in every human bile analysed, chenodeoxycholic acid constitutes more than 30 % of the total bile acid. This relatively high proportion of chenodeoxycholic acid still seems to be characteristic of human bile.

SUMMARY

1. A series of eight primate-bile-salt specimens has been analysed. The method combined a countercurrent procedure, which separated glycine and taurine conjugates, and chromatography controlled by infrared spectroscopy.

2. Within experimental error, the glycine-conjugated and taurine-conjugated fractions contained the same bile acids in similar proportions.

3. The major constituent acids in all the bile samples were chenodeoxycholic acid (3 α :7 α -dihydroxycholanolic acid), deoxycholic acid (3 α :12 α -dihydroxycholanolic acid) and cholic acid (3 α :7 α :12 α -trihydroxycholanolic acid). Only in human bile does chenodeoxycholic acid occur as more than 30 % of the total acids.

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