0·80–0·85 % P, were free from detectable impurities as shown by fractional solubility tests, electrophoretic examination and behaviour in the analytical ultracentrifuge at pH 7·0.

6. Specific polysaccharide dissociates progressively from the antigen at alkaline pH values.

7. Sedimentation constants at pH's 4·5 and 8 obtained for the phospholipid-free antigen and extrapolated to infinite dilution give approximately the same figure (0·007), which indicates a particle weight of the order of 1×10^7.

8. The material possesses an LD_{50} of 80 μg. in the mouse and induces formation of 'Shiga' agglutinins in rabbits on the injection intravenously of 1 μg.

We wish to thank Dr K. Smith, Lister Institute Research Student (1945–48), for much help in the earlier part of this investigation and Dr B. R. Record, of the Microbiological Research Department, Porton, for freeze-drying facilities and for electrophoretic and ultracentrifugal examinations. One of the authors (W.M.) thanks the Stiftung für Stipendien auf dem Gebiete der Chemie, Bern (1948–50) for financial support. Permission to publish that part of the work carried out at M.R.D. has been granted by the Chief Scientist, Ministry of Supply.

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Comparative Studies of 'Bile Salts'

7. BILE ACIDS OF THE COYPU, *MYOCASTOR COYPUS*

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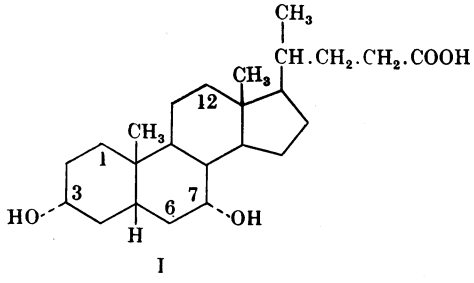
Myocastor coypus is a vegetarian rodent of South American origin which, bred for its fur (nutria), has become acclimatized in the wild state in this country and elsewhere in Europe (Laurie, 1946). Brigl & Benedict (1933) reported that the bile of this animal contained a unique bile acid, 'nutriacholic acid', conjugated with glycine; this claim was supported by Haslewood & Wootton (1950). Prof. Shimizu of Okayama University, in a letter to the present author dated February 1952, stated that Kazuno in 1946 had shown that 'nutria glycocholic acid' was a conjugate of 3-hydroxy-7-oxocholanic acid and glycine, and that coypu bile also contained ursodeoxycholic acid (3α:7β-dihydroxycholanic acid). At that time work on coypu bile in these laboratories had been continued and it was decided to attempt a comprehensive study of the bile acids. At the same time, the opportunity was to be taken of testing new methods for the purification and identification of such substances.

RESULTS

It was at once found that Kazuno's claim was well founded; 'nutriacholic acid' was identical with 3α-hydroxy-7-oxocholanic acid (III), prepared in good yield by partial oxidation of chenodeoxycholic acid (I). It was also found that condensation of this ketonic substance, as its ethyl ester, with Girard's reagent 'T' was not quantitative; nine separate Girard processes were required to extract it almost completely from the mixed ethyl esters obtained from the bile salts. In the non-ketonic material left after these experiments, esters of the following were identified: cholic (3α:7α:12α-trihydroxycholanic) acid, chenodeoxycholic (3α:7α-dihydroxycholanic) acid (I) and ursodeoxycholic (3α:7β-dihydroxycholanic) acid (II); the isolation of derivatives of these substances was greatly facilitated by the paper chromatography described below, and the ethyl esters of the last two compounds were actually first

separated and isolated in milligram quantities by this method. Cholic acid constituted less than 1 % of the bile acids, and the isolated mixture of bile acids is thought to have been made up almost entirely of the three other bile acids identified, present in comparable proportions.



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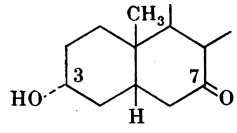
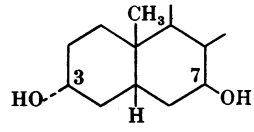


III



II

With the help of Prof. W. R. Spurrell, an experiment was carried out to try to discover whether the bile of the coypu really contained 'nutriacholic acid' produced by the liver. A drainage tube was inserted into the hepatic duct of a live animal under anaesthesia, the blood coming from the intestine was cut off and bile was collected after a period judged to be probably long enough to clear the circulating blood of products of microbial action in the gut. Such bile was found to contain a quantity of 'nutriacholic acid', and paper chromatography suggested that it also contained chenodeoxycholic and ursodeoxycholic acids.

Ethyl esters of the six possible hydroxyoxocholanic acids derived by oxidation to ketone of one or two hydroxyl groups of cholic acid (Haslewood, 1946) as well as ethyl 3α-hydroxy-7-oxocholanate and ethyl 12α-hydroxy-3-oxocholanate were tested on paper for their reaction with Kritchevsky & Kirk's (1952*a*) phosphomolybdic acid reagent; only ethyl 3α-hydroxy-7:12-dioxocholanate and 3α-hydroxy-7-oxocholanate failed to give a blue colour (compare Kritchevsky & Kirk, 1952*a*, and Sjövall, 1952).

Preparation of, and experiments with, crystalline ethyl hyodeoxycholate (ethyl 3α:6α-dihydroxycholanate) showed that this substance and ethyl ursodeoxycholate behaved in a closely similar fashion on paper chromatograms.

EXPERIMENTAL

General. Melting points were determined on a Kofler block type of apparatus and are corrected. $Al_2O_3(S)$ was 'Type H' (Peter Spence, Widnes); $Al_2O_3(N)$ was from Messrs Hopkin and Williams, neutralized as described by Shoppee (1949). Light petroleum had b.p. 40–60°, unless otherwise stated. Microanalyses (C and H) were by Drs Weiler and Strauss, Oxford.

Purification of bile acids

Hydrolysis of bile salts. Coypu bile salts (1 g., prepared as described by Haslewood & Wootton, 1950) were heated at about 110° for 6·5 hr. in a sealed metal bomb with water (5 ml.) and 5N-NaOH (5 ml.). Acidification of the diluted contents of the bomb gave a brown solid which was collected, washed and dried *in vacuo* over H_2SO_4. Yield, 5·66 g. from 9 g. of bile salts.

Esterification of bile acids. The above material (5·66 g.) was dissolved at about 23° in ethanol (60 ml.) containing H_2SO_4 (1·2 ml.). After 3 days the mixture was diluted with a solution of $NaHCO_3$ (in excess) in water. The oily neutral product was extracted with ether and the ether was washed with water, dried over Na_2SO_4 and evaporated. The residual ethyl esters weighed 5·9 g.

Girard separation of ethyl esters. The above product (5·9 g.), after being dried *in vacuo* over H_2SO_4, was heated under reflux for 1 hr. with ethanol (60 ml.) containing acetic acid (6 ml.) and Girard's reagent 'T' (2 g.). The cooled mixture was diluted, adjusted to a pH of about 7 with NaOH soln. and the 'non-ketones' were extracted with ether in the usual way. Adjustment of the aqueous residue to about 2N with HCl gave, after 16 hr. at about 25°, the 'ketones'. These were extracted with ether and the ether was washed with dilute NH_3 soln. and water, dried over Na_2SO_4 and evaporated. Acidification of the NH_3-water washings gave the 'ketonic acid' fraction (Table 1, column 3) evidently formed by hydrolysis during the above processes. This was in all cases identified by m.p. and mixed m.p. determinations as 3α-hydroxy-7-oxocholanic acid.

The 'non-ketones' from the above process were again separated as before, and this was repeated for a total of nine separations, of which the results are shown in Table 1. In this table, the working loss (column 5) was material not accounted for as the sum of the 'non-ketonic' and ketonic fractions; if there were no loss, this sum should be equal to the 'non-ketonic' fraction from the previous separation.

Table 1. *Course of Girard separations of ethylated 'bile acids' (5·9 g.) from coypu bile*

No. of Girard separations	Material separated (g.) 'Non-ketones'	'Ketones' Acidic	'Ketones' Total	Estimated working loss (g.)
1	5·091	Not collected	0·644	0·165
2	4·623	0·065	0·408	0·060
3	3·982	0·120	0·435	0·206
4	3·725	0·054	0·185	0·072
5	3·513	Nil	0·191	0·021
6	3·410	0·025	0·089	0·014
7	3·335	0·024	0·057	0·018
8	3·290	0·012	0·044	0·001
9	3·229	0·005	0·018	0·043
		Total	2·071	0·600

Table 2. *Chromatography of non-ketonic esters from coypu bile on alumina*

Esters, 3·229 g.; Al_2O_3, 70 g. in a column (28 × 2 cm.).

Fraction no.	Eluted by: Solvent	Eluted by: Vol. (ml.)	Description: Wt. (g.)	Description: Properties
I	Benzene	105	0·235	Oil
II and III	Benzene	100	0·128	Oil
IV	Benzene	100	0·004	Oil
V–IX	10, 10, 20, 30, 50% (v/v) ether/benzene	100 for each fraction	Traces	—
X	Ether	50	Trace	—
XI	5% (v/v) ethanol/ether	50	Trace	—
XII (*a*)	10% (v/v) ethanol/ether	50	Trace	—
XII	10% (v/v) ethanol/ether	20	1·526	Yellow gum
XIII	10% (v/v) ethanol/ether	25	0·844	Gum
XIV and XV	10% (v/v) ethanol/ether	100	0·090	Gum
XVI–XVIII	10% (v/v) ethanol/ether	100 for each fraction	0·060	Gum
XIX–XXII	Increasing amounts of ethanol in ether; finally ethanol	50–100 for each fraction	Few mg.	Gum
XXIII	10% (v/v) acetic acid/ethanol	150	0·220	Partially acidic yellow gum
		Total recovered	3·107	

Table 3. *Chromatography of combined ester Fractions XII and XIII, from the separation shown in Table 2, on alumina*

Esters, 2·370 g.; Al_2O_3, 25 g.

Fraction	Eluted by: Solvent	Eluted by: Vol. (ml.)	Description: Wt. (g.)	Description: Properties
A	Benzene	30	Nil	—
B	Benzene	30	1·357	Yellow gum
C	Benzene	10	0·126	Gum
D	Benzene	10	0·073	Gum
E	Benzene	20	0·075	Gum
F	Benzene	20	0·039	Gum
G	Benzene	40	0·043	Gum
H–M	Benzene	600	0·173	Gum
N	Benzene	1000	0·087	Yellow gum
O	Ether	100	0·122	Yellow gum
P	Ethanol	60	0·249	Yellow gum
	Total recovered		2·344	

Treatment of ketonic fractions. The first three ketonic fractions extracted were examined by paper chromatography as described below. Only very small amounts of material giving a colour with the spraying reagent were detected. All the ketonic esters from the separations were therefore combined and this material (1·67 g.) was hydrolysed by boiling under reflux in ethanol (20 ml.) with aqueous KOH (2 ml. of 40%, w/v) for 30 min. The clear product was cooled, diluted, and acidified with HCl. The partly crystalline acids were collected, washed with water and crystallized from ethyl acetate. Two successive crops of crystals weighed 1·19 and 0·15 g. and had melting points of 199–201° and 194–197°, respectively. This material was identical in crystalline form, m.p. and mixed m.p. with authentic 3α-hydroxy-7-oxocholanic acid prepared from chenodeoxycholic acid (p. 586). Evaporation of the final ethyl acetate mother liquors gave a gum (0·162 g.) which, after re-esterification, still showed only faint spots on paper chromatography; it was not further investigated.

Treatment of non-ketonic fractions. A solution of the final fraction (3·229 g.: Table 1) in benzene was percolated through a column (28 × 2 cm.) of Al_2O_3 (*S*, 70 g.). Fractions were eluted as shown in Table 2. Fractions XIV–XXII inclusive were examined by paper chromatography (see p. 584). Fractions XVI–XXI each gave a spot moving only a short distance from the starting line, as well as one with an R_F of about 0·5 (e.g. Fig. 1 *A*). The former spot was shown to run at the same rate as an ethyl cholate and the latter as an ethyl chenodeoxycholate standard. Fractions XVII and XVIII became partially crystalline on standing with light petroleum; the gummy crystals were recrystallized from light petroleum/benzene. Crops from XVII and XVIII had melting points 156–158° and 159–162°, respectively, not depressed by admixture with ethyl cholate. The crystals gave a blue colour in the Hammarsten HCl test. No ethyl cholate spot showed on paper chromatography of fractions XIV, XV or XXII; hence the maximum amount of ethyl cholate which could have been present in the non-ketones was about 100 × 0·06/3·229, i.e. about 1·9%, and was probably less than half this quantity. There was thus considerably less than 1% of cholic acid in the coypu bile acids examined. Fractions I–III and also XXII were not further examined.

The main fractions, i.e. XII and XIII, were combined, dissolved in benzene and separated on Al_2O_3 (*N*, 25 g.) on a column, with the results shown in Table 3. Fractions *A–O* were each examined by paper chromatography and all showed spots corresponding to ethyl chenodeoxycholate. However, in certain fractions, notably *D*, *E* and *F*, a second spot was seen, running at about half the rate of ethyl chenodeoxycholate (Fig. 1, *B*, *C*). Fractions *D–F* were combined, hydrolysed in the usual way and an attempt was made to separate the acids by partition chromatography as described by Bergström & Sjövall (1951). This was unsuccessful, but J. Sjövall subsequently showed, in the author's laboratory, that a separation was possible with a 10% (v/v) heptane:chloroform/55% (v/v) methanol:water system (see Sjövall, 1952). The esters were separated by paper chromatography; that giving the upper spot was ethyl chenodeoxycholate and that giving the lower spot was ethyl ursodeoxycholate (see pp. 584, 585).

Fraction *B* (Table 3) was examined by infrared spectroscopy by Mr H. Wiggins, in Dr I. D. P. Wootton's laboratory (Wootton, 1953): it was reported to consist of ethyl chenodeoxycholate with another component, not ethyl deoxycholate.

A solution of fraction *P* (0·249 g.) in ether was washed successively with dilute HCl, water, dilute NH_3 soln. and water. The ether was dried over Na_2SO_4 and evaporated, and the residue (0·244 g.) was examined by infrared spectroscopy as above and by paper chromatography. The examination by infrared spectroscopy suggested that it consisted very largely of ethyl chenodeoxycholate and only one spot, corresponding to this, was seen on the paper. However, it is felt that some substance(s) of a different type may have been present. The impression was gained that all the fractions from Al_2O_3 (Table 3) contained ethyl chenodeoxycholate, but that ethyl ursodeoxycholate was present also in considerable amounts in most fractions. It constituted the greater proportion in fractions *D*–*F* (see p. 585).

Paper chromatography of bile acids and their esters

Sjövall (1952), Kritchevsky & Kirk (1952*b*) and Beyreder & Rettenbacher-Däubner (1953) have described methods different from the following.

General methods. Use was made of the solvent systems of Bush (1952) and of the 10% (w/v) phosphomolybdic acid-in-ethanol, spraying reagent of Kritchevsky & Kirk (1952*a*). Strips of Whatman 3MM paper were ruled with the starting line at 8–10 cm. from one end. When the ethanolic solutions of substances to be run had been applied at the starting line (0·5 cm. diam. spots) and allowed to dry, the paper was hung in a glass tank so that the starting line was at the bottom. The tank was now tilted and the moving phase was added so as to be contained in the lower part of the tank; the paper was prevented from reaching this phase by a beaker containing the stationary phase. This stationary phase was allowed to run up a narrow strip or strips of filter paper. The tank was sealed with (*a*) a cloth moistened with stationary phase, (*b*) a cellophan cover and (*c*) a rubber cover, all tightly held by rubber bands. At least 16 hr. were allowed for equilibration, after which the tank was carefully tilted so as to allow the moving phase to reach the loaded paper. When the solvent front had reached a distance of about 20–22 cm. above the starting line, the paper was removed, dried in air and at about 90°, sprayed and heated for about 2 min. at 100° to bring up the blue spots. As the temperature was not controlled within 20–25°, R_F values were not reliable (Bush, 1952), and standard solutions (1–10 mg./ml. in ethanol) on the same paper were used to identify spots. The only difficulty encountered was that, when there had been wide variations in temperature, the stationary phase sometimes condensed on the tank walls and then ran on to the paper when the tank was re-tilted after equilibration. If this caused serious trouble, the experiment could be stopped before the solvent front had reached the starting line, the paper briefly dried in air and the whole re-assembled with fresh samples of the phases. Equilibration in these circumstances only required about 2 hr.

Results. The following could easily be separated, using the systems of Bush (1952) mentioned in brackets: cholic, deoxycholic or chenodeoxycholic, hyodeoxycholic and lithocholic acids (*B* 5); ethyl cholate, trihydroxycoprostanate (Haslewood, 1952), deoxycholate or chenodeoxycholate, and hyodeoxycholate (*A*). Deoxycholic and chenodeoxycholic acids and their methyl or ethyl esters were not separable by these methods (see also Sjövall, 1952). Opportunity was taken to test the reactions of the following, as spots (containing about 30 μg.) on paper: the six possible ethyl hydroxyoxocholanates derived by oxidation to ketone of one or two hydroxyl groups in ethyl cholate (Haslewood, 1946); ethyl 3α-hydroxy-7-oxocholanate and ethyl 12α-hydroxy-3-oxocholanate. A blue colour was given by all except ethyl 3α-hydroxy-7:12-dioxocholanate and ethyl 3α-hydroxy-7-oxocholanate. 3α-Hydroxy-7:12-dioxo-, 3α-7α-dihydroxy-12-oxo- and 3α-hydroxy-7-oxocholanic acids were tested by Sjövall (1952); the present results agree with his for the parent acids. Lithocholic acid and its ethyl ester also gave a rather faint colour, not detected by Sjövall (1952). Sjövall reported that 3α-hydroxy-12-oxocholanic acid gave no colour with the reagent.

Separation of ethyl chenodeoxycholate and ethyl ursodeoxycholate on paper. Pieces of Whatman 3MM paper about 30 × 22 cm. were ruled across the longer axis to make a starting line 6·5 cm. from one end. This line (22 cm. long) was marked with dots at 0·5 cm. intervals. A solution (66 mg. in 3·3 ml. of ethanol) of the mixed ethyl esters obtained in fractions *D*–*F* (Table 3) was allowed to fall, from a graduated pipette drawn out into a fine capillary, on to these dots so as to make a confluent row of spots. The amount on the paper (about 5 mg.) was known by reading the pipette. A large battery jar served as a tank for each paper and the separations were carried out exactly as described above, with Bush's system *A*. When the papers had been dried, strips 0·5 cm. in width were cut from a long edge and sprayed; the appearance is shown in Fig. 2. These sprayed strips were laid beside the rest of the paper, as they had been cut, and the unsprayed paper was ruled across so that the transverse zones (shown by the strips) occupied by the two separated substances could be cut out separately. Fourteen such separations were done involving almost all the material (66 mg.). The combined 'upper' and 'lower' zones cut from the papers as described above were extracted separately in a Soxhlet apparatus with methanol. Evaporation of the methanol left, in each case, a yellowish gum, which was further purified by passage in benzene through a column containing Al_2O_3 (*N*, 0·5 g.). The recovery was: from the combined upper zones—22 mg.; from the combined lower zones—40 mg.; total, 62 mg. It was calculated that unavoidable losses amounted to about 4 mg.; hence recovery was quantitative. Mr Wiggins, by infrared spectroscopy, found that the material from the upper zone was ethyl chenodeoxycholate and from the lower zone was a hitherto unexamined substance, converted on chromic oxidation into ethyl dehydrochenodeoxycholate (3:7-dioxocholanate). On paper chromatography, material from each zone gave only a single spot, in their expected positions (Fig. 3). The lower zone material could not be ethyl hyodeoxycholate (spectroscopic evidence) although the spot on paper from it (Fig. 4) corresponded to this substance. It was hydrolysed, and the acid obtained crystallized readily from dilute ethanol on seeding with authentic ursodeoxycholic acid, the gift of Prof. T. Shimizu. Shimizu's ursodeoxycholic acid melted at 202–204°, the acid from the lower zone at 203–204·5° and the mixture at 202–204°.

Material from the upper zone (20 mg.) was dissolved in acetic acid (0·5 ml.) with acetic anhydride (0·15 ml.). 6 N-$HClO_4$ (1 drop) was added and, after 25 min., the mixture was diluted and extracted with ether. The ether was washed with water, NH_3 soln., and water, then dried over Na_2SO_4

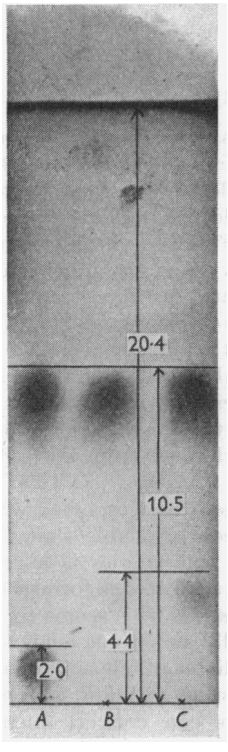


Fig. 1.

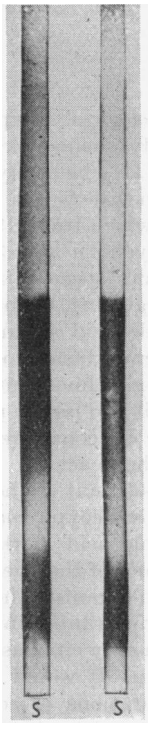


Fig. 2.

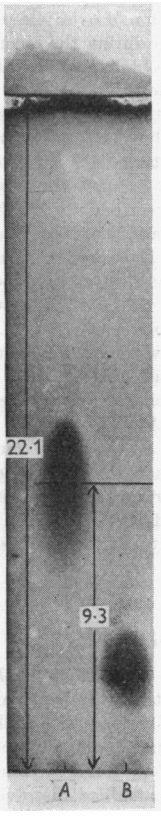


Fig. 3.

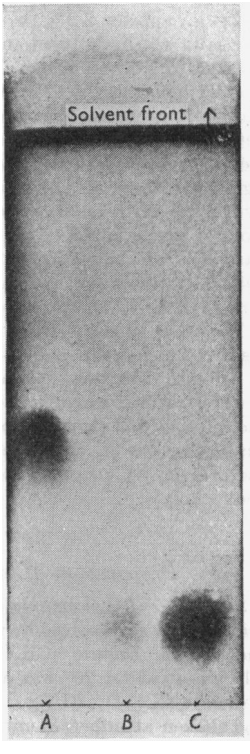


Fig. 4.

Fig. 1. Paper chromatogram of fractions separated on Al_2O_3 of bile acid esters from the coypu. *ABC*, starting line. *A*, fraction XVI (Table 2); *B* and *C*, mixed fractions *D–F* (Table 3). Solvent system *A* (Bush, 1952).

Fig. 2. Strips cut from sheets of paper used to separate mixed fractions *D–F* (Table 3) from bile acid esters from coypu bile. *S*, *S*, portions of starting line. Upper and lower zones (dark) show positions of separated substances on whole paper.

Fig. 3. Paper chromatogram of substances separated into zones as shown in Fig. 2 and described in text. *AB*, starting line. *A*, from upper zone (ethyl chenodeoxycholate); *B*, from lower zone (ethyl ursodeoxycholate). Solvent system *A* (Bush, 1952).

Fig. 4. Paper chromatogram of purified ethyl esters of bile acids. *ABC*, starting line. *A*, ethyl chenodeoxycholate; *B*, ethyl hyodeoxycholate; *C*, ethyl ursodeoxycholate. Solvent system *A* (Bush, 1952).

and evaporated. The residue, in light petroleum, was boiled with charcoal and the filtered solution evaporated. The colourless residue crystallized on standing at about 5° with a little light petroleum, giving large, colourless crystals of m.p. 102–106°, not depressed by ethyl diacetylchenodeoxycholate (m.p. 104–106°).

It was concluded that the combined fractions *D–F* (186 mg., Table 3) contained ethyl chenodeoxycholate (about 62 mg.) and ethyl ursodeoxycholate (about 124 mg.). Ethyl ursodeoxycholate and ethyl hyodeoxycholate ran at virtually the same rate on paper (Fig. 4) and both substances gave a sluggish and rather faint reaction with the spraying reagent.

Collection and treatment of bile from a live coypu

Collection. A coypu about 5 years old (senile) and weighing about 9 lb., the gift of Mr G. Iles, was anaesthetized with Nembutal. The abdomen was opened and the gall bladder was tied off, emptied of bile with a syringe and removed. A cannula was put into the hepatic duct and the portal vein was tied. Bile flowing from the liver (at the rate of 4–6 ml./hr.) was collected during 3 hr., after which the animal died. The operative procedures were carried out by Prof. W. R. Spurrell.

Treatment of bile. The gall-bladder bile (16·5 ml.) was diluted with ethanol (50 ml.) and the filtered solution was

evaporated to give the bile salts (0·638 g.; approx. 3·9% (w/v) of the bile). The first hour's collection of liver bile was discarded and the bile (11 ml.) collected during the second and third hours was treated as above to obtain the bile salts (0·224 g.; approx. 2·2% (w/v) of the bile). Each sample of bile salts was hydrolysed and the resulting bile acids were isolated and esterified as described above. Yields: gall-bladder bile esters, 0·34 g.; liver bile esters, 0·056 g. Each sample of esters was separated once into ketones and 'non-ketones' with Girard's reagent 'T' as described above. The yields of ketones for a single separation cannot be considered significant (see p. 581), but each ketonic fraction gave, after hydrolysis, on crystallization from ethyl acetate and then from dilute ethanol, a high yield of colourless leaflets. Samples from gall-bladder and liver bile both had m.p. 195–197°, unchanged by admixture with an authentic sample of 3α-hydroxy-7-oxocholanic acid.

The 'non-ketones' from the liver bile (0·042 g.) were dissolved in benzene and run through Al_2O_3 (*N*, 0·5 g.) in a column. Benzene (60 ml.) eluted a colourless gum (0·027 g.) which was dissolved in ethanol (0·2 ml.). When the solution was examined by paper chromatography with Bush's system *A*, spots were obtained corresponding to those given by ethyl chenodeoxycholate and ethyl ursodeoxycholate.

Preparation of bile-acid derivatives

3α-Hydroxy-7-oxocholanic acid (III). Chenodeoxycholic acid (0·1 g.) was dissolved by warming with acetic acid (1 ml.) and sodium acetate ($CH_3CO_2Na.3H_2O$; 0·2 g.). To the solution at about 20° was added a solution of potassium chromate (K_2CrO_4, 31·7 g./100 ml.; 0·15 ml.). The mixture was shaken at intervals until the chromate had dissolved (about 30 min.) and was then left with occasional shaking at about 20° for 6 days, during which time crystals separated. Dilution with water gave a crystalline solid which was collected, washed and recrystallized from ethyl acetate, from which the above acid separated as colourless prisms (0·06 g.; 60%) of m.p. 195–197°. *Ethyl 3α-hydroxy-7-oxocholanate*, prepared in the usual way and crystallized from a small quantity of light petroleum, formed white needles of m.p. 61–63°. (Found: C, 74·2; H, 9·9. $C_{26}H_{42}O_4$ requires C, 74·6; H, 10·1%.) *Ethyl 3α-acetoxy-7-oxocholanate*, prepared by acetylation in the usual way of the above ester, formed colourless needles from light petroleum and white leaflets from dilute ethanol; it had m.p. 120–121°. (Found: C, 73·2; H, 9·5. $C_{28}H_{44}O_5$ requires C, 73·0; H, 9·6%.) This substance closely resembled ethyl 3α:7α-diacetoxycholanate (ethyl diacetyl chenodeoxycholate) in appearance and behaviour, and crystals of either compound could be used successfully to 'seed' preparations and solutions of the other to induce crystallization.

Ethyl hyodeoxycholate. A solution of hyodeoxycholic acid (0·1 g.) in ethanol (2 ml.) containing H_2SO_4 (0·04 ml.) was left at about 20° for 4 days. The diluted mixture was stirred with $NaHCO_3$ (in excess) and the oily product extracted with ether. The ether was washed with water, dried over Na_2SO_4 and evaporated. The gummy residue was purified by passage in benzene through Al_2O_3 (*N*, 1 g.) and the product left after evaporation of the benzene eluate crystallized on standing with light petroleum. *Ethyl hyodeoxycholate (ethyl 3α:6α-dihydroxycholanate)* crystallized from light petroleum/benzene as long, white needles of m.p. 114–116°. (Found: C, 74·2; H, 10·4; $C_{26}H_{44}O_4$ requires C, 74·3; H, 10·5%.)

Ethyl ursodeoxycholate (ethyl 3α:7β-dihydroxycholanate). This was prepared in a similar fashion, but showed no tendency to crystallize.

DISCUSSION

Biological. Coypu bile has been shown to contain chiefly chenodeoxycholic acid and substances (II and III) which may be considered to be derived from it. In this respect, it resembles the bile of the guinea pig, which Imai (1937) has shown to contain chenodeoxycholic and 3α-hydroxy-7-oxocholanic acids. In both species the conjugation is almost entirely with glycine, although in the coypu a small quantity of bile acid conjugated with taurine probably is present (Haslewood & Sjövall, 1954). Other species showing almost entirely glycine conjugation are the rabbit and pig; in neither of these cases can the bile acids be considered to be derived from chenodeoxycholic acid.

The result of the experiment now reported with the live coypu makes it seem probable that the ketonic acid in the bile of this species is a true product of the liver and not a substance formed by micro-organisms in the intestine. This seems to be an important point, especially as ketonic acids are frequently cited as intermediates in bile acid metabolism. It would be interesting to know whether preparations of coypu liver can convert chenodeoxycholic acid or its glycine conjugate into the 7-oxo derivative. Hoehn, Schmidt & Hughes (1944) showed that oxidation (to ketone) at position 7 could be readily brought about in cholic acid by a strain of *Acaligenes faecalis* and, of course, analogous oxidations both by micro-organisms and by tissue preparations are well known in steroids other than bile acids. There is therefore nothing extraordinary in the idea that a ketonic acid should be a major constituent of the bile acids in a species, although this seems to be a somewhat unusual occurrence.

The finding of ursodeoxycholic acid in the coypu destroys the view that this substance is characteristic of the bile of bears. Earlier history of work on this acid was discussed by Haslewood & Wootton (1950) who concluded that bear's bile contained cholic acid as its chief constituent. The results of paper chromatography on the liver bile esters from the coypu suggested that ursodeoxycholic acid may be made by the liver itself in this species.

One result of Kazuno's and the present investigation is to remove all grounds for the use of the name 'nutriacholic acid', which is neither an acid unique in coypu bile nor a cholic (trihydroxycholanic) acid. Coypu bile is in fact 'unique' in the same sense as guinea pig bile and the relationship

may possibly indicate more general biological affinities.

It is tempting to suppose that the small amount of cholic acid now shown to be present in *Myocastor coypus* represents a vestigial 'memory' of a previous time in which cholic was (as in most mammals today) the chief bile acid; this view implies that it is more recent evolution which has produced the 'glyco-dihydroxy' type of bile salt discussed above.

Chemical. The present work puts beyond doubt the superiority of methods of partition chromatography over the hitherto usual separations on alumina for investigations of mixtures of bile acids. Complete separation of the dihydroxy esters clearly would not have been possible without partition methods. The isolation after paper chromatography described here was easy to carry out and could be applied in all instances where more than one spot can be obtained. Separation on alumina remains a valuable method of handling material, especially in larger quantities, for preliminary purification.

A working hypothesis which will explain the (at present) known results of testing ketonic bile acids and esters with the phosphomolybdic acid reagent is the following. In cholanic acid derivatives containing oxygen at positions 3, 7, 12 or any one or two of these, at least one hydroxyl group in the α position is needed to reduce the reagent; an α-hydroxyl group at C-3 only will not give a reaction in the presence of carbonyl groups at C-7, C-12 or both positions. An α-hydroxyl at C-7 or C-12 will reduce the reagent irrespective of carbonyl groups at one or both of the remaining positions considered. Such rules, if confirmed, may be of value in the study of constitutional problems; they are clearly capable of considerable amplification.

The resistance of ethyl 3α-hydroxy-7-oxocholanate to reaction with Girard's reagent 'T' was not altogether unexpected, for Hoehn *et al.* (1944) had already noticed that 3α:12α-dihydroxy-7-oxocholanic acid 'does not form a Girard hydrazone'.

SUMMARY

1. An investigation of the chemical nature of the bile acids of the coypu, *Myocastor coypus*, has been carried out. In agreement with Kazuno, it has been found that the 'nutriacholic acid' of Brigl & Benedict (1933) is 3α-hydroxy-7-oxocholanic acid, and that coypu bile contains ursodeoxycholic (3α:7β-dihydroxycholanic) acid. Chenodeoxycholic (3α:7α-dihydroxycholanic) and cholic (3α:7α:12α-trihydroxycholanic) acids have now also been found in the bile of this species. The name 'nutriacholic acid' should no longer be used.

2. Examination of bile collected from a living coypu suggested that 3α-hydroxy-7-oxocholanic and ursodeoxycholic acids may be primary products of the liver and not artifacts absorbed from the intestine.

3. Some biological implications of the nature of coypu bile have been briefly discussed.

4. Methods of paper chromatography have been used to trace and to isolate the above bile acids; such methods were more effective than chromatography on alumina alone.

5. Ethyl 3α-hydroxy-7-oxocholanate was resistant to reaction with Girard's reagent 'T', and nine extractions were necessary to remove it effectively from the mixture of ethylated bile acids.

6. The ethyl esters of eight hydroxy-keto bile acids, substituted at two of, or all, the positions 3, 7 and 12 of the cholanic acid molecule have been tested, as spots on paper, with the phosphomolybdic acid reagent of Kritchevsky & Kirk (1952*a*). The results, together with those previously reported by other workers, can be explained by a simple working hypothesis.

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