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Further Studies on the Formation of Bile Acids in the Guinea Pig.

Bile Acids and Steroids 141

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The conversion of 4-^{14}C-cholesterol to bile acids was studied in two strains of guinea pigs, ranging in weight from 250 to 960 g. It was not possible to demonstrate the formation of cholic acid in these animals. Lithocholic acid was identified and ursodeoxycholic acid tentatively identified as minor components of guinea pig bile. Two unidentified neutral steroids, compounds I and II, were isolated from bile. Compound II was converted mainly into chenodeoxycholic acid in the bile fistula rat.

In an earlier communication from this laboratory Danielsson and Kazuno [1] reported on the conversion of cholesterol to bile acids in the guinea pig. Chenodeoxycholic acid was found to be the principal bile acid formed in the liver. The 3α-hydroxy-7-keto-5β-cholanic acid present in gall bladder bile appeared to represent mainly a product of microbial oxidation of chenodeoxycholic acid during the enterohepatic circulation of bile, as this acid was absent from fistula bile collected later than 6—8 h after establishment of a bile fistula. No evidence was obtained to indicate that cholesterol was converted into a trihydroxycholanic acid. This latter finding was in accord with previous knowledge of the composition of the bile acids in guinea pig bile as reported by Imai.[2]

More recently, however, Peric-Golia and Jones [3,4] have isolated cholic acid from guinea pig bile and shown that it is formed from cholesterol in the liver. Interestingly enough, cholic acid was present in bile of mature guinea pigs only and not in bile of immature guinea pigs. The failure of Danielsson and Kazuno to demonstrate the conversion of cholesterol to a trihydroxycholanic acid in the guinea pig could then be explained by the immaturity of the animals used by these authors. Peric-Golia and Jones [4] also demonstrated that 3α-hydroxy-7-keto-5β-cholanic acid can be formed from cholesterol in the liver and thus does not arise only by microbial oxidation of chenodeoxycholic acid.

The finding of Peric-Golia and Jones that cholic acid appears in guinea pig bile only upon maturation of the animals seemed of such interest as to warrant further work on the formation of bile acids in this species. In an attempt to study the mechanisms underlying this phenomenon an investigation of bile acid formation in guinea pigs of different weights was undertaken. However, no evidence for the formation of cholic acid was obtained with the two strains of guinea pigs available. In addition to these results, the present communication describes the identification of lithocholic acid, the tentative identification of ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanic acid), and the isolation of two unknown neutral steroids from guinea pig bile.

EXPERIMENTAL

Guinea pigs of two different strains (Danish State Serum Institute and the Swedish National Defence Laboratory) were used. The animals were fed a commercial chow diet (Anticimex, Stockholm, Sweden). Bile fistulas were prepared by introducing a polyethylene cannula into the common bile duct. An amount of saline solution or bile equal to the volume of bile excreted through the fistula was supplied continuously through a stomach fistula.

Cholesterol-4-^{14}C (Radiochemical Centre, Amersham, England; 60 μC per mg), 10–50 μC as a serum albumin stabilized emulsion, was injected intraperitoneally at different times prior to or after operation of the animals.

Analytical procedures. Bile was collected in ethanol for 2–5 days in 12 h portions. Conjugated bile acids were extracted with butanol from the acidified bile filtrate. Bile was hydrolyzed at 110° for 8–12 h with N sodium hydroxide in 50 % aqueous ethanol. The acidified hydrolysate was extracted with ether.

For column chromatographic separations phase system C 1 was used for conjugated bile acids [5] and phase systems F 1 and F 2 for free bile acids.[5] Paper chromatographic analyses of free and conjugated bile acids were performed with phase systems described by Sjövall.[6,7] For thin layer chromatography of bile acids phase systems described by Eneroth [8] were employed. Neutral steroids were separated on columns of aluminum oxide, grade III (Woelm, Eschwege, W.-Germany). Autoradiography of thin layer chromatograms was performed as described by Norman and Palmer.[9]

RESULTS

Formation of bile acids. The conversion of 4-^{14}C-cholesterol to bile acids was studied in 26 guinea pigs, ranging in weight from 250 to 960 g. The labeled cholesterol (10–50 μC) was administered in a single dose 24 or 120 h before or 24 h after establishment of a bile fistula. In the first 12 h portions of bile from animals injected with cholesterol prior to operation an average of 45 % of the labeled bile acids excreted was conjugated with glycine as analyzed by column and paper chromatography. The labeled bile acids excreted in subsequent bile portions were conjugated almost exclusively with taurine. When labeled cholesterol had been administered after operation, all labeled bile acids excreted were present as taurine conjugates.

The composition of the free bile acids was analyzed in all instances by column chromatography and in some cases also by paper and thin layer chromatography. No consistent differences in bile acid composition were observed between immature and mature animals injected with labeled cholesterol prior to operation. The time, 24 or 120 h, that had elapsed between administration of labeled cholesterol and operation, appeared to have no

marked influence on the composition of the labeled bile acids. Fig. 1 shows the chromatograms of the first 12 h portions of bile from four animals, weighing 250, 580, 660, and 960 g, respectively. The main part of the radioactivity eluted from the columns coincides with the titration peak of chenodeoxycholic acid (40—70 ml of effluent). The second largest radioactive peak corresponds to 3α-hydroxy-7-keto-5β-cholanic acid (70—100 ml of effluent). Smaller amounts of radioactivity were eluted as two peaks at 12 to 18 ml and 25 to 35 ml of effluent, respectively. Approximately 10 % of the radioactivity put on the columns was retained in the stationary phase. In subsequent bile portions the amount of radioactive 3α-hydroxy-7-keto-5β-cholanic acid decreased markedly. On an average, this acid accounted for 18 % of the labeled bile acids in the first 12 h portions as compared with 6 % in the following bile portions. In animals injected with labeled cholesterol 24 h after operation the composition of the labeled bile acids excreted in bile differed in some respects. The relative amount of labeled 3α-hydroxy-7-keto-5β-cholanic acid was less, constituting on an average 4 % in the first 12 h portions and 5 % in subsequent bile portions. No significant quantities of radioactivity appeared in the 25—35 ml effluent region.

Since the first radioactive peak in the chromatograms shown in Fig. 1 appeared in the vicinity of the position characteristic of cholic acid, further investigation of the nature of this radioactive material appeared desirable. Such material from several bile portions was combined and chromatographed together with unlabeled cholic acid on phase system C 1. Irrespective of the weight of the animals from which these samples originated, very small amounts of radioactivity were eluted within the titration peak of cholic acid. Upon crystallization with additional unlabeled cholic acid most of this radioactivity was recovered from the mother liquors. The main part of the radioactivity eluted from these columns appeared earlier than cholic acid. About 60 % of the radioactivity put on these columns was retained in the stationary phase.

Tentative identification of ursodeoxycholic acid. The labeled material eluted between 25 and 35 ml of effluent in the chromatograms shown in Fig. 1 appears at a place characteristic of ursodeoxycholic acid. Such material from several bile portions was combined and was found to have exactly the same chromatographic properties as authentic ursodeoxycholic acid in paper and thin layer chromatography. The labeled compound could not be crystallized and sufficient amounts of unlabeled ursodeoxycholic acid were not available to allow a confirmatory identification by crystallization to constant specific activity.

Identification of lithocholic acid. The radioactive material recovered from the stationary phase of the columns shown in Fig. 1 was rechromatographed together with unlabeled chenodeoxycholic acid using phase system F 2 (*cf.* Fig. 2). A small part of the radioactivity eluted coincided with the titration peak of the added chenodeoxycholic acid, whereas the main part appeared later at a position characteristic of lithocholic acid. Thin layer chromatography demonstrated that this labeled compound was indistinguishable from lithocholic acid and the identity of the labeled compound with lithocholic acid was confirmed by crystallization to constant specific activity after addition of unlabeled lithocholic acid.

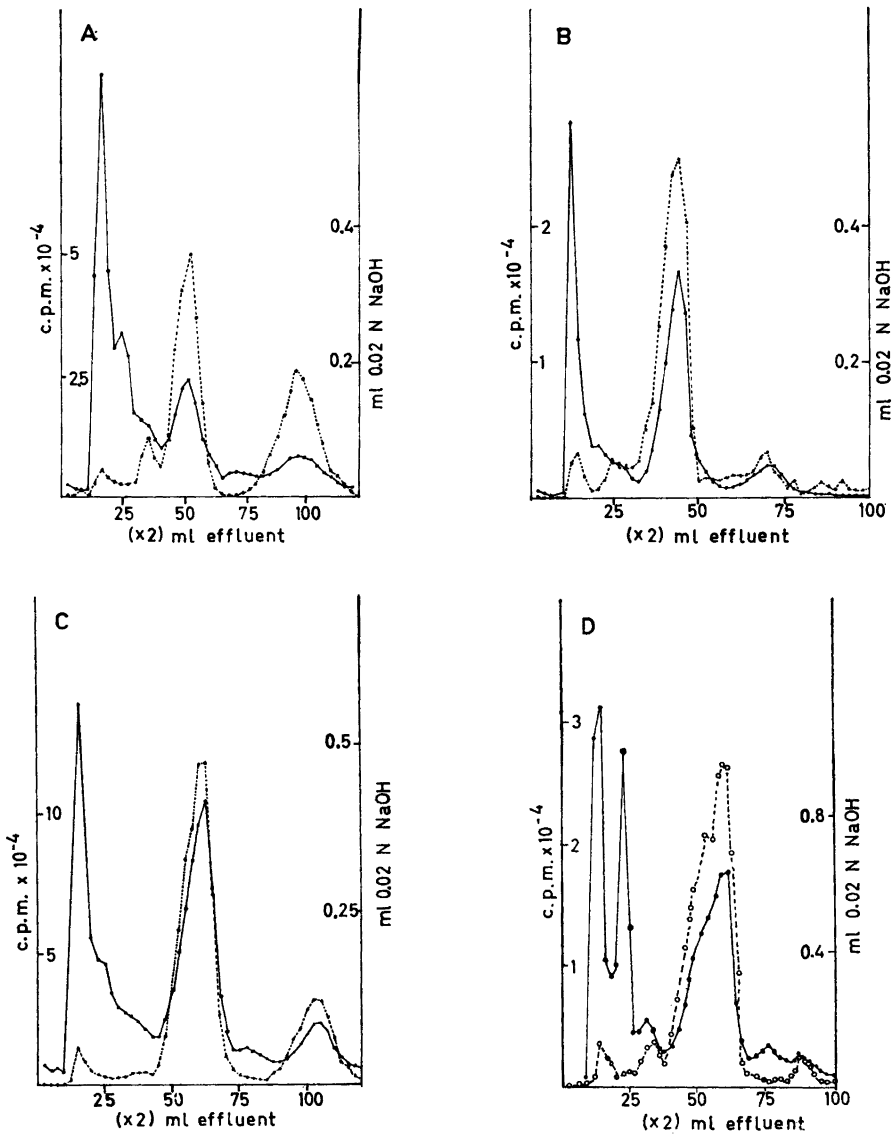

Fig. 1. Chromatograms of the first 12 h portions of hydrolyzed bile obtained from guinea pigs injected with 4-^{14}C-cholesterol prior to operation. Curve A, guinea pig weighing 250 g, injected 120 h before operation. Curve B, guinea pig weighing 580 g, injected 24 h before operation. Curve C, guinea pig weighing 660 g, injected 120 h before operation. Curve D, guinea pig weighing 960 g, injected 24 h before operation. Columns: 9 g of hydrophobic Hyflo Super Cel. Phase system: F 1. Broken line: radioactivity. Solid line: titration values.

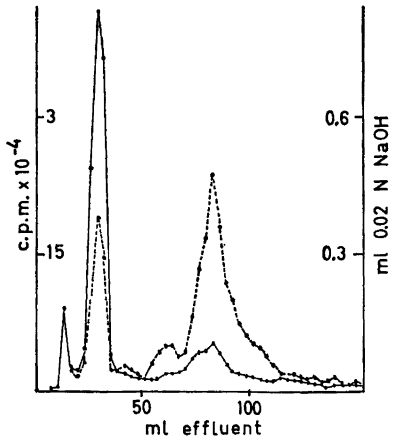

Fig. 2. Chromatogram of alcohol eluate of the columns shown in Fig. 1. Column: 4.5 g of hydrophobic Hyflo SuperCel. Phase system: F 2. Broken line: radioactivity. Solid line: titration values.

Isolation of two unknown neutral steroids. The alcohol eluate of the column shown in Fig. 2 was chromatographed on a column of 20 g of aluminum oxide, grade III, together with 10 mg of cholesterol and 16 mg of 7α-hydroxycholesterol, *cf.* Fig. 3. Three major labeled compounds were present, one coinciding with cholesterol (fractions 8—12), one (compound I) appearing shortly before the peak of 7α-hydroxycholesterol (fractions 37—45), and one (compound II) after. Compounds I and II comprised about 4 % of the total radioactivity

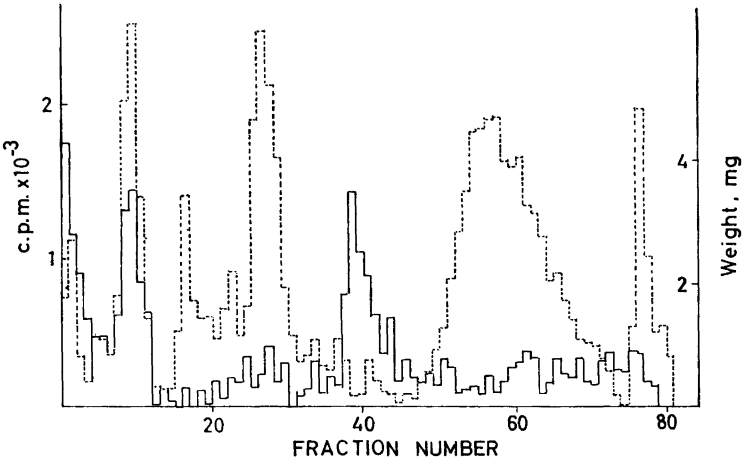

Fig. 3. Chromatogram of alcohol eluate of column shown in Fig. 2. Column: 20 g of aluminum oxide, grade III. Eluting solvents: H = hexane; B = benzene; E = ethyl acetate; M = methanol. Fractions (20 ml each): 1—4 = H/B, 3:7; 5—8 = H/B, 2:8; 9—12 = H/B, 1:9; 13—16 = B; 17—25 = B/E, 9:1; 26—32 = B/E, 8:2; 33—36 = B/E, 7:3, 37—40 = B/E, 6:4; 41—44 = B/E, 5:5; 45—50 = B/E, 4:6; 51—58 = B/E, 3:7; 59—70 = B/E, 2:8; 71—74 = B/E, 1:9; 75—76 = E; 77—79 = E/M, 1:9; 80—81 = M. Broken line: radioactivity. Solid line: weight.

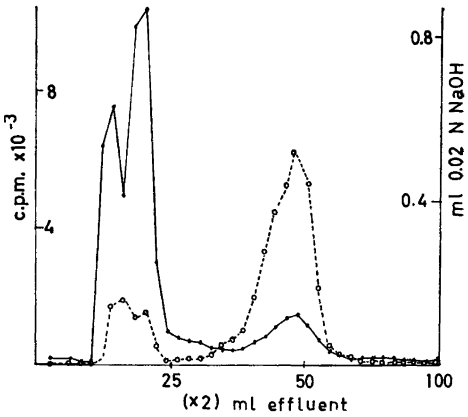

Fig. 4. Chromatogram of first 24 h portion of hydrolyzed bile from bile fistula rat injected with compound II (55 000 c.p.m.). Column: 9 g of hydrophobic Hyflo Super-Cel. Phase system: F 1. Broken line: radioactivity. Solid line: titration values.

excreted in bile. The structures of these compounds have not been established. The metabolism of compound II in the bile fistula rat was investigated using methods recently described.[10] This compound was found to be metabolized mainly into chenodeoxycholic acid and to a smaller extent into compounds with chromatographic mobilities in phase system C 1 similar to those of α- and β-muricholic acids (3α,6β,7α-trihydroxy-5β-cholanic and 3α,6β,7β-trihydroxy-5β-cholanic acids). Less than 10 % of the radioactivity excreted in bile appeared within the titration peak of cholic acid. Fig. 4 shows a chromatogram with phase system F 1 of hydrolyzed bile from a bile fistula rat injected with compound II. The major part of the radioactivity coincides with the titration peak of chenodeoxycholic acid (35—55 ml of effluent). Due to the lack of sufficient amounts of labeled compound I the metabolism of this compound in the bile fistula rat could not be examined.

DISCUSSION

The present investigation on the formation of bile acids in immature and mature guinea pigs has failed to demonstrate the conversion of cholesterol to cholic acid and it has not been possible to isolate cholic acid from gall bladder bile. The discrepancy between these results and those of Peric-Golia and Jones [3,4] is evidently explained by differences in the strains of guinea pigs used. In this connection, a report by Haslewood [11] is of interest. He found in the bile of the coypu, a rodent related to the guinea pig, that cholic acid is present only in small quantities, constituting less than 1 % of the bile acids, whereas chenodeoxycholic, ursodeoxycholic, and 3α-hydroxy-7-keto-5β-cholanic acids were the main bile acids. In agreement with the findings of Peric-Golia and Jones [4] 3α-hydroxy-7-keto-5β-cholanic acid is formed also in the liver of the guinea pig, presumably by oxidation of chenodeoxycholic acid. Haslewood [11] obtained evidence indicating that in the coypu 3α-hydroxy-7-keto-5β-cholanic acid was a primary bile acid formed in the liver.

The present investigation has led to the tentative identification of ursodeoxycholic acid and the identification of lithocholic acid as minor components

of guinea pig bile. It is probable that ursodeoxycholic acid arises by reduction of 3α-hydroxy-7-keto-5β-cholanic acid in the liver. In a previous report [1] it was shown that 3α-hydroxy-7-keto-5β-cholanic acid is reduced also to chenodeoxycholic acid which is the predominant product of this reaction. In the species so far examined [12-14] lithocholic acid has been shown to be formed from chenodeoxycholic acid by the action of intestinal microorganisms. It would therefore seem likely that this is the case also in the guinea pig.

The isolation of two unknown neutral steroids from guinea pig bile is described in the present report. Both of these compounds were more polar than cholesterol, compound I showing chromatographic properties indicating the presence of one keto and one hydroxyl group or two hydroxyl groups, whereas compound II had chromatographic properties similar to those of 7α-hydroxycholesterol. Compound II was converted mainly into chenodeoxycholic acid in the bile fistula rat. Attempts to determine the structures of these unknown steroids are in progress.

Addendum. Recently we had an opportunity to examine by paper chromatography bile from guinea pigs (weighing from 200 to 700 g) of an English short hair strain delivered by local suppliers around Urbana, Ill. With the methods used no cholic acid could be detected in bile from these animals.

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