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On the Formation of Cholic Acid in the Bile Fistula Rat from Some Naturally Occurring Polyhydroxy Bile Sterols

Bile Acids and Steroids 143

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The extent of conversion to cholic acid in the bile fistula rat of 5β-bishomocholane-3α,7α,12α,24-tetrol, 5β-bishomocholane-3α,7α,12α,24,26-pentol, 5β-cholestane-3α,7α,12α,25,26-pentol, and 5β-cholestane-3α,7α,12α-triol was studied. All of these compounds were converted to cholic acid but in widely varying yields. The results were discussed in relation to current concepts of the mechanism of conversion of cholesterol to bile acids.

The mechanism of conversion of cholesterol to bile acids has been studied mainly in mammalian systems.[1] From this work it has been concluded that in the formation of cholic acid the hydroxyl groups at the C-7 and C-12 positions are introduced prior to the oxidation of the side-chain. The mechanism of oxidation of the side-chain has been postulated as an ω-oxidation followed by a "β"-oxidation with the release of propionic acid. This concept is based mainly on results of studies of the metabolism of 5β-cholestane-3α,7α,12α-triol *in vitro*. The initial reaction in the ω-oxidation has been shown to be a hydroxylation at the C-26 position.[2] The substrate for the 26-hydroxylase in cholic acid formation has not been identified conclusively but has been suggested to be 5β-cholestane-3α,7α,12α-triol. A recent report by Mendelsohn and Staple[3] lends strong support to this contention. The conversion of cholesterol to cholic acid in mammals would thus include the following steps: cholesterol→7α-hydroxycholesterol→5β-cholestane-3α,7α,12α-triol→3α,7α,12α-trihydroxy-5β-cholestanoic acid→cholic acid + propionic acid.

In bile of more primitive animals, *e.g.* sharks, amphibians, and certain reptiles, the main bile salts so far identified are bile alcohols and bile acids having 27 carbon atoms.[4] These compounds are all derived from cholesterol. Haslewood[4–6] has found that the composition of the bile salts of an animal often can be correlated with its position in the evolutionary series. He has

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further proposed that the mechanism of conversion of cholesterol to C_{24} bile acids in mammals is a recapitulation of the evolution of bile salts and thus would entail the intermediary formation of C_{27} steroids similar to or the same as those found in bile of lower species. The main evidence so far obtained in favor of this concept concerns 3α,7α,12α-trihydroxy-5β-cholestanoic acid. This acid, which is the main bile acid in bile of frogs, toads and crocodiles, has been shown to be converted efficiently into cholic acid in the bile fistula rat.[6] In addition, it has been isolated from human bile[7] and its formation from cholesterol in human liver has also been demonstrated.[8]

Recently, Kazuno and collaborators[9–11] have isolated 5β-bishomocholane-3α,7α,12α,24-tetrol and 5β-bishomocholane-3α,7α,12α,24,26-pentol from bull frog bile and 5β-cholestane-3α,7α,12α,25,26-pentol from toad bile. These sterols have been shown to be formed from cholesterol.[12–13] The main bile acid in bull frog as well as in toad bile is 3α,7α,12α-trihydroxy-5β-cholestanoic acid. Small amounts of cholic acid are present in bull frog bile,[14] but cholic acid has not been isolated from toad bile. The interesting question then arises if the C_{26} bile sterols in bull frog bile are intermediates in the conversion of cholesterol to cholic acid in the bull frog and in mammals. If this were the case there would exist an alternative pathway of cholic acid formation in which 3α,7α,12α-trihydroxy-5β-cholestanoic acid would be decarboxylated to a C_{26} sterol. This, in turn, would be hydroxylated at the C-26 and/or the C-24 positions to yield cholic acid by as yet unknown reactions. Similarly, the structure of 5β-cholestane-3α,7α,12α,25,26-pentol isolated from toad bile might be an indication of an additional alternative pathway of cholic acid formation. However, it is also possible that this compound is a result of a side-reaction not involved in the evolution of cholic acid. In this connection it is of interest that scymnol (5β-cholestane-3α,7α,12α,24,26,27-hexol[15–16]), the main sterol in bile of sharks, is an inefficient precursor of cholic acid in the rat as could be expected from its structure and also in view of the fact that sharks represent, phylogenetically, the development of an early side-branch of the path of evolution leading to mammals.[17]

In an attempt to answer these questions the metabolism of tritium labeled 5β-bishomocholane-3α, 7α, 12α,24-tetrol, 5β-bishomocholane-3α, 7α, 12α, 24, 26-pentol, and 5β-cholestane-3α,7α,12α,25,26-pentol was studied in the bile fistula rat. For the sake of comparison the metabolism of 5β-cholestane-3α,7α,12α-triol was also studied under the same experimental conditions.

EXPERIMENTAL

Tritium labeling. Labeling of the compounds with tritium was carried out according to the procedure of Wilzbach [18] in the apparatus described by Bergström and Lindstedt.[19] Labeled 5β-cholestane-3α,7α,12α-triol was prepared by Kolbe electrolysis of tritium labeled cholic acid and unlabeled isovaleric acid,[20] followed by extensive purification by chromatography on columns of aluminum oxide, grade IV (Woelm, Eschwege, W.-Germany). The same amount, 2 mg, of 5β-bishomocholane-3α,7α,12α,24-tetrol, 5β-bishomocholane-3α,7α,12α,24,26-pentol, and 5β-cholestane-3α,7α,12α,25,26-pentol was exposed at the same time in the same reaction vessel to 2 C of tritium gas (Radiochemical Centre, Amersham, England) for 2 weeks. The compounds were purified by repeated chromatography on 4.5 g columns of hydrophobic Hyflo SuperCel using phase system F 3 (methanol/water, 150/150 ml; chloroform/heptane, 45/5 ml) for 5β-bishomocholane-3α,7α,12α,24-

tetrol and phase system C 2 for the other two sterols.[21] The total amount of tritium in the three compounds was: 12 μC in 5β-bishomocholane-3α,7α,12α,24-tetrol, 707 μC in 5β-bishomocholane-3α,7α,12α,24,26-pentol, and 43 μC in 5β-cholestane-3α,7α,12α,25,26-pentol.

Animal experiments. Bile fistula rats were prepared in the usual manner using male rats of the Sprague—Dawley strain weighing about 250 g. A few μC of labeled sterol were mixed with 1 mg of respective unlabeled sterol and injected intraperitoneally as a serum albumin stabilized emulsion. After injection of the sterol the bile was collected in 24 h portions.

Analysis of bile. Bile was acidified and extracted with water-saturated butanol. The butanol extract was washed with water until neutral and evaporated to dryness under reduced pressure. The residue was chromatographed on 4.5 g of hydrophobic Hyflo SuperCel using phase system C 1.[21] The early part of the effluent (10—35 ml of effluent) containing taurocholic and taurochenodeoxycholic acids was combined and hydrolyzed in sealed steel bombs with N sodium hydroxide in 50 % aqueous ethanol for 8—12 h at 110°. The hydrolysate was acidified and extracted three times with ether. The combined ether extracts were washed with water until neutral and evaporated to dryness. The residue was chromatographed as described above using phase system C 1. Paper chromatography was performed as described by Sjövall [22] using phase system F_a and F_{am}. The latter system is a modification of F_a consisting of a change of the mobile phase to 60 % ethylene chloride. Scanning of radioactivity on paper chromatograms was performed with a non-commercial gas-flow counter.

RESULTS

Excretion of radioactivity in bile. In view of the large differences in amount of tritium incorporated into the different sterols during exposure to tritium gas, it was of importance to measure accurately the amounts of radioactivity excreted in bile after injection of the sterols. In all cases very small amounts of radioactivity were excreted in the second 24 h portions of bile. The percentage of administered isotope excreted in bile in the first 24 h portion was for 5β-bishomocholane-3α,7α,12α,24-tetrol 80 and 83 %; for 5β-bishomocholane-3α,7α,12α,24,26-pentol 34, 34, 38, 38, and 39 %; for 5β-cholestane-3α,7α,12α,25,26-pentol 51, 60, 64, and 68 %; for 5β-cholestane-3α,7α,12α-triol 57, 59, and 63 %.

Analysis of labeled products in bile. Fig. 1 shows the chromatograms of the first 24 h portions of bile from four animals injected with, respectively, 5β-bishomocholane-3α,7α,12α,24-tetrol (curve A), 5β-bishomocholane-3α,7α,12α,24,26-pentol (curve B), 5β-cholestane-3α,7α,12α,25,26-pentol (curve C), and 5β-cholestane-3α,7α,12α-triol (curve D). The amount of radioactivity eluted within the taurocholic-taurochenodeoxycholic acid band (10—35 ml of effluent) varied considerably. In the case of 5β-cholestane-3α,7α,12α-triol (curve D) almost all of the radioactivity eluted from the column appeared within this band. About 20 % of the radioactivity applied to the column was retained in the stationary phase and was found to consist of unchanged 5β-cholestane-3α,7α,12α-triol, 3α,7α,12α-trihydroxy-5β-cholestanoic acid, and one unknown compound.

The main part of the radioactivity derived from 5β-bishomocholane-3α,7α,12α,24-tetrol (curve A) was eluted after the taurine-conjugated bile acids and about 25 % of the total radioactivity applied to the column was retained in the stationary phase. The labeled material appearing between 35 and 65 ml of effluent was chromatographed on paper using phase system F_a and was shown to consist of several unknown compounds. Using the same procedure

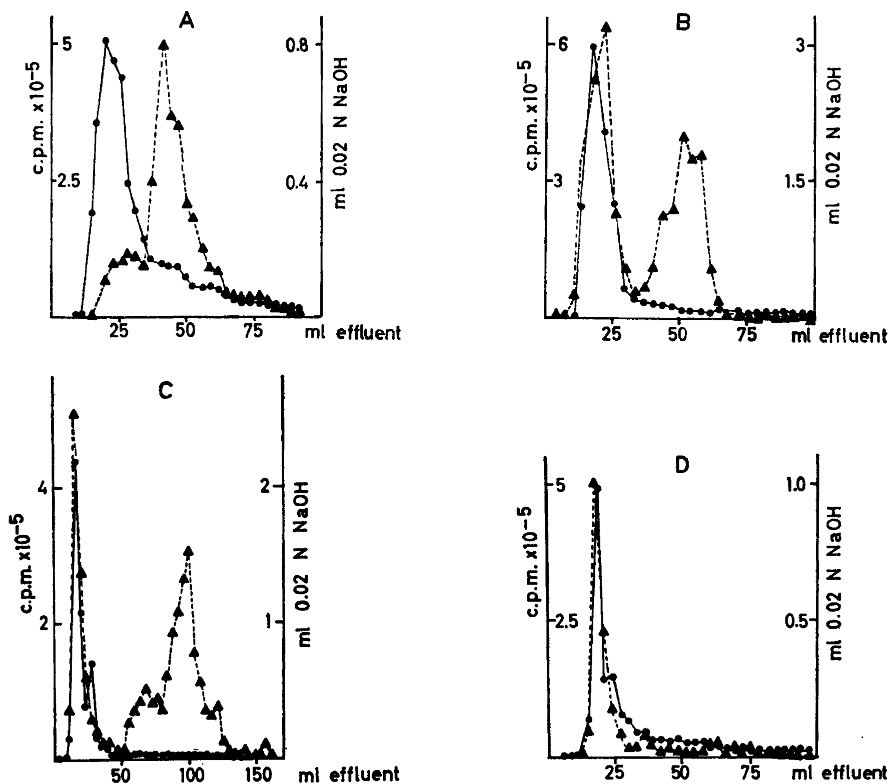

Fig. 1. Chromatograms of first 24 h portions of bile from bile fistula rats injected with, respectively, 5β-bishomocholane-3α,7α,12α,24-tetrol (curve A), 5β-bishomocholane-3α,7α,12α,24,26-pentol (curve B), 5β-cholestane-3α,7α,12α,25,26-pentol (curve C), and 5β-cholestane-3α,7α,12α-triol (curve D). Columns: 4.5 g of hydrophobic Hyflo SuperCel. Phase system: C 1. Broken line: radioactivity. Solid line: titration values.

small amounts of 5β-bishomocholane-3α,7α,12α,24-tetrol were detected in the labeled material retained in the stationary phase.

The radioactivity excreted in bile after administration of 5β-bishomocholane-3α,7α,12α,24,26-pentol (curve B) was eluted in two main peaks, the second peak appearing between 35 and 65 ml of effluent. About 20 % of the total radioactivity was retained in the stationary phase and was found to be a mixture of several unknown compounds. Paper chromatography with phase system F_{am} of the labeled material eluted between 35 and 65 ml of effluent showed that it consisted of starting material and smaller amounts of one unknown compound.

When 5β-cholestane-3α,7α,12α,25,26-pentol was given, the major part of the radioactivity was eluted between 55 and 130 ml of effluent (curve C), and consisted mainly of unchanged 5β-cholestane-3α,7α,12α,25,26-pentol as shown by paper chromatography with phase system F_{am}. About 10 % of

the total radioactivity applied to the column was retained in the stationary phase.

The taurocholic-taurochenodeoxycholic acid bands from above-mentioned chromatograms were hydrolyzed and chromatographed with phase system C 1 (*cf.* Fig. 2). As is seen in Fig. 2 almost all of the radioactivity originating from 5β-bishomocholane-3α,7α,12α,24,26-pentol (curve F), 5β-cholestane-3α,7α,12α,25,26-pentol (curve G), and 5β-cholestane-3α,7α,12α,-triol (curve H)

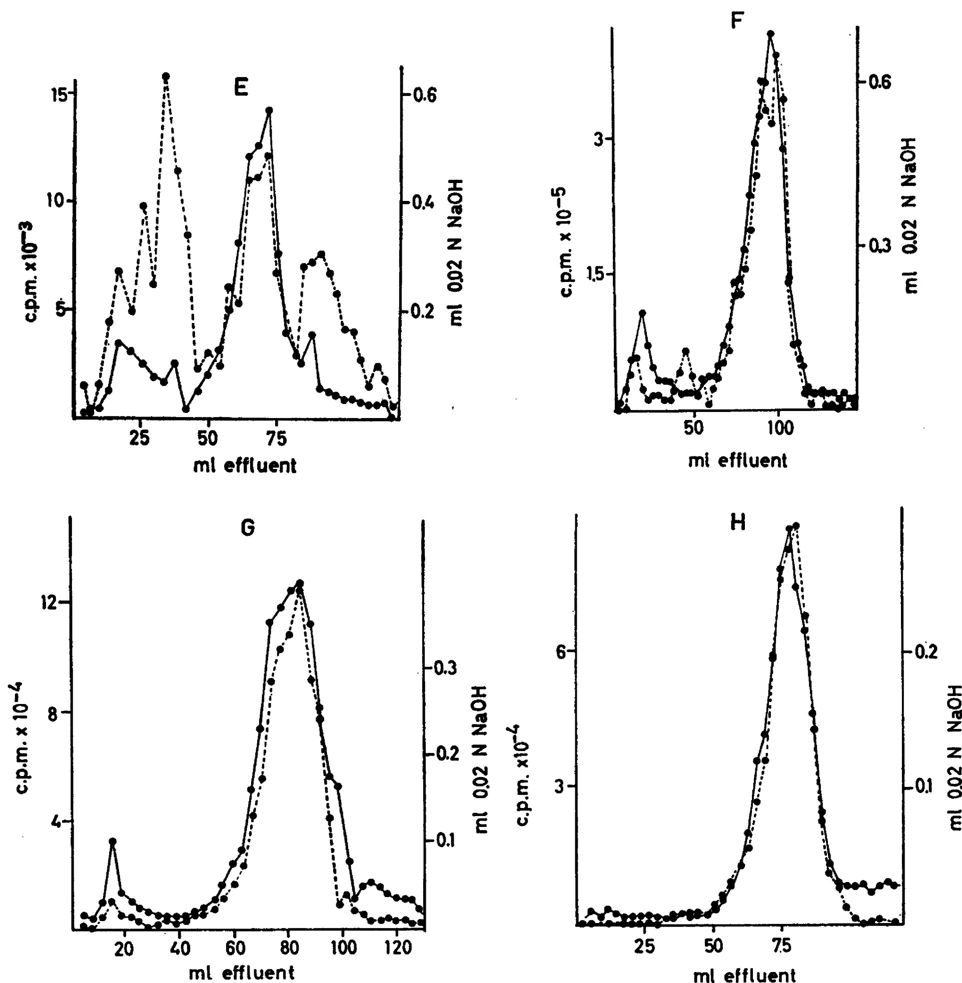

Fig. 2. Chromatograms of hydrolyzed taurocholic-taurochenodeoxycholic acid bands (*cf.* chromatograms shown in Fig. 1) derived from, respectively, 5β-bishomocholane-3α,7α,12α,24-tetrol (curve E), 5β-bishomocholane-3α,7α,12α,24,26-pentol (curve F), 5β-cholestane-3α,7α,12α,25,26-pentol (curve G), and 5β-cholestane-3α,7α,12α-triol (curve H). Columns: 4.5 g of hydrophobic Hyflo SuperCel. Phase system: C 1. Broken line: radioactivity. Solid line: titration values.

coincided with the titration peak of cholic acid. The identity of the radioactivity with cholic acid was established in each case by crystallization to constant specific activity after dilution with unlabeled cholic acid. In the case of 5β-bishomocholane-3α,7α,12α,24-tetrol (curve E) only part of the radioacitivity coincided with the titration peak of cholic acid. This labeled material was shown to be identical with cholic acid by crystallization to constant specific acitivity.

DISCUSSION

The compounds studied in the present investigation were all converted to cholic acid in the bile fistula rat but in widely varying yields. Of the total radioacitivity excreted in the first 24 h portions of bile, cholic acid accounted for about 80 % when 5β-cholestane-3α,7α,12α-triol had been administered. The corresponding figures for the other three sterols were about 5 % of cholic acid from 5β-bishomocholane-3α,7α,12α,24-tetrol, about 40 % from 5β-bishomocholane-3α,7α,12α,24,26-pentol, and about 25 % from 5β-cholestane-3α,7α,12α,25,26-pentol. To provide a meaningful comparison of these figures the distribution of the tritium label in the molecules must be considered. In the case of 5β-cholestane-3α,7α,12α-triol the tritium was confined to the "cholic acid moiety" of the molecule and should not be lost during oxidation of the side-chain. The average recovery of radioactivity in bile was 60 % for this sterol. The fate of the tritium not accounted for is not known. Similar recoveries of radioactivity in bile have been obtained when other C_{27} sterols labeled with tritium by the same method, *i.e.* coupling of a tritium labeled bile acid with unlabeled isovaleric acid, have been administered. The highest recovery of radioactivity in bile was obtained with 5β-bishomocholane-3α,7α,12α,24-tetrol. This sterol was not metabolized to C_{24} steroids to any appreciable extent and the value obtained for the amount of cholic acid formed could not be too much in error due to loss of tritium during oxidation of the side-chain. However, in the case of 5β-bishomocholane-3α,7α,12α,24,26-pentol only 38 % of the radioactivity administered was recovered in bile. Apparently, a considerable amount of the tritium in this sterol was located in the side-chain and was lost in the oxidation of the side-chain. This is also evident from the extent of incorporation of tritium in this compound during exposure to tritium gas (*cf.* experimental section). The value obtained for the extent of conversion of 5β-bishomocholane-3α,7α,12α,24,26-pentol to cholic acid is therefore a minimum value and the conversion to cholic acid is more probably around 80 % than 40 %. In the case of 5β-cholestane-3α,7α,12α,25,26-pentol about 60 % of the radioactivity administered was recovered in bile. This figure is the same as that obtained with 5β-cholestane-3α,7α,12α-triol labeled in the "cholic acid moiety". However, it cannot be concluded on this ground that no isotope was lost during oxidation of the side-chain. If the location of tritium in 5β-cholestane-3α,7α,12α,25,26-pentol was similar to that in 5β-bishomocholane-3α,7α,12α,24-tetrol, 20 % of the tritium could have been lost during oxidation of the side-chain and the extent of conversion of cholic acid of 5β-cholestane-3α,7α,12α,25,26-pentol might therefore be somewhat higher than the 25 % found.

Of the compounds studied 5β-cholestane-3α,7α,12α-triol was most efficiently converted to cholic acid. The mechanism of formation of cholic acid

from this compound entails an ω-oxidation followed by a "β"-oxidation with release of propionic acid. The extent of conversion to cholic acid of 5β-bishomocholane-3α,7α,12α,24,26-pentol was of the same order of magnitude. In this case the mechanism of side-chain oxidation is not known but probably entails an oxidation of the C-26 hydroxyl group to a carboxyl group followed by a β-oxidation. Whether these reactions are part of an alternative pathway of cholic acid formation, *i.e.* one with C_{26} intermediates, or represent lack of specificity of the enzyme systems involved in the oxidation of the C_{27} side-chain cannot be stated at present. The yield of cholic acid from 5β-cholestane-3α,7α,12α,25,26-pentol was considerably less. The mechanism of cholic acid formation from this compound is not known, but such a pathway is apparently less important, quantitatively. The inefficient conversion of 5β-bishomocholane-3α,7α,12α,24-tetrol to cholic acid demonstrates that the terminal methyl group of the side-chain is the first to be oxidized and 5β-bishomocholane-3α,7α,12α,24-tetrol is apparently a result of a side-reaction not involved in the degradation of cholesterol to cholic acid. Shimizu *et al.*[23] have reached a similar conclusion in a study of the formation of cholic acid from 5β-cholestane-3α,7α,12α,24-tetrol.

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