Conversion of Δ^5-Cholestene-3α-12α-diol to Cholic Acid in the Rabbit

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The work of Bergström and collaborators (*cf.* Ref.[1]) has demonstrated that during the conversion of cholesterol to cholic acid the steroid nucleus is modified prior to the completion of the side-chain oxidation. Studies of the metabolism of cholesterol and other C_{27}-neutral sterols in liver mitochondrial preparations have shown that the major metabolic event is the introduction of a hydroxyl-group at position 26. The metabolites formed, *e.g.* 26-hydroxycholesterol from cholesterol [2], Δ^5-cholestene-3β,7α,26-triol from 7α-hydroxycholesterol [3], coprostane-3α,7α,26-triol from coprostane-3α,7α-diol [7], are converted mainly to chenodeoxycholic acid when administered to bile-fistula rats. None of the metabolites isolated from these *in vitro* experiments appears to contain a 12α-hydroxyl-group. It seems apparent that the introduction of the 12α-hydroxyl-group which is a necessary step to form the cholic acid nucleus, is a reaction that is not easily reproduced *in vitro*. The nature of the substrate for the 12α-hydroxylase is not entirely known. The work of Lindstedt [5] on the metabolism of 7α-hydroxycholesterol in the bile-fistula rat has suggested the possibility that the formation of 7α-hydroxycholesterol is one of the early steps in the degradation of cholesterol to bile acids. There are, however, no definite reasons to exclude the possibility that the introduction of the 12α-hydroxyl-group could be one of the early steps.

In view of the results of the *in vitro* experiments mentioned above and as a prequisite for *in vitro* studies it seemed of interest to examine the metabolism of 12α-hydroxycholesterol *in vivo*. In a recent communication [6] the synthesis of Δ^5-cholestene-3β,12α-diol, *i.e.* 12α-hydroxycholesterol, was described and the metabolism of this compound in the bile-fistula rat was reported. It was found that 12α-hydroxycholesterol was converted to deoxycholic acid, cholic acid, and one so far unidentified metabolite which was somewhat more polar than deoxycholic acid. As rat liver is known to contain a 7α-hydroxylase which converts deoxycholic acid to cholic acid [7], these results do not unequivocally show that cholic acid is formed directly from 12α-hydroxycholesterol. Hence, it was necessary to repeat these experiments in an animal known not to contain an enzyme that converts deoxycholic acid to cholic acid. Lindstedt and Sjövall [8] have shown that the rabbit is not able to convert deoxycholic acid to cholic acid and the rabbit is therefore the experimental animal of choice for an investigation of the possibly direct formation of cholic acid from 12α-hydroxycholesterol. This communication reports the formation of cholic acid and deoxycholic acid from 12α-hydroxycholesterol in the bile-fistula rabbit.

Experimental. White rabbits, weighing approximately 2.5 kg, were used. Under nembuthal anaesthesia a polyethylene tubing was introduced into the lower part of the common bile duct. Bile was collected in 24 h portions. The bile was saponified in sealed steel tubes with 1 N sodium hydroxide in 50 % ethanol at 110° for 12 h. The free bile acids were obtained by ether extraction of the saponification mixture and were separated by reversed phase chromatography with phase system F 1 [9]. The trihydroxycholanic acid band from these chromatograms were rechromatographed with

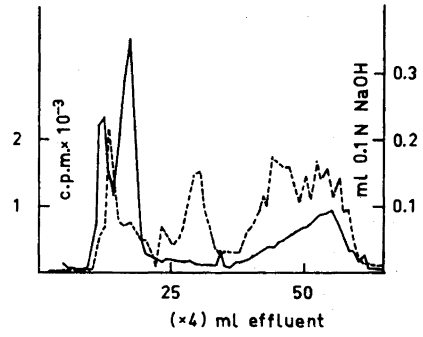

Fig. 1. Chromatography of first 24 h portion of hydrolyzed bile from bile-fistula rabbit injected with tritium labeled 12α-hydroxycholesterol. Column: 18 g hydrophobic Hyflo. Phase system F 1.
Solid line: titration values. Broken line: radioactivity.

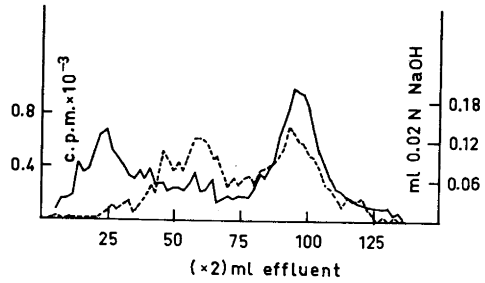

Fig. 2. Rechromatography of the cholic acid band, cf. Fig. 1.
Column: 9 g hydrophobic Hyflo. Phase system C 1.
Solid line: titration values. Broken line radioactivity.

phase system C 1 [9]. The tritium labeled Δ^5-cholestene-3β,12α-diol used was the same material as that described in an earlier paper [6].

Results and discussion. After intraperitoneal administration of a serum albumin stabilized emulsion of Δ^5-cholestene-3β,12α-diol (3 and 6 mg, resp.) 21 and 18 %, resp., of the injected dose was excreted in bile during the subsequent 48 h. Fig. 1 shows a chromatogram of the first 24 h portion of hydrolyzed bile from one rabbit. Three main radioactive bands are seen: one, apparently composed of at least two compounds, is eluted in the vicinity of the titration peak of cholic acid, which has its maximum at 16 ml of effluent; the second radioactive peak appears at 30 ml of effluent and the third is eluted together with deoxycholic acid which has its peak at 55 ml of effluent. Approximately 70 % of the radioactivity eluted together with deoxycholic acid was shown to be identical with deoxycholic acid by crystallization to constant specific radioactivity. The identity of the radioactive material eluted with its maximum at 30 ml of effluent, is not known. A compound with the same chromatographic properties was also formed from 12α-hydroxycholesterol in the rat. The cholic acid band, appearing between 10 and 20 ml of effluent (Fig. 1.) was rechromatographed on a column with phase system C 1 (*cf.* Fig. 2). Approximately half of the radioactivity put on the column was eluted together with cholic acid and the identity of the radioactive material with cholic acid was established by crystallization to constant specific radioactivity. The radioactive material eluted before cholic acid has not been identified. Of the radioactivity excreted in bile after administration of 12α-hydroxycholesterol about 40 % could be accounted for as deoxycholic acid and about 10 % as cholic acid.

The results of this and the previous investigation on the metabolism of 12α-hydroxycholesterol in the bile-fistula rat [6] are summarized in Fig. 3. The conversion of 12α-hydroxycholesterol to cholic acid in the bile-fistula rat is more efficient than what is observed in the rabbit, but in the case of the rat there is an enzyme in the liver that is capable of converting deoxycholic acid to cholic acid. It is therefore not possible to conclude that there is a path-

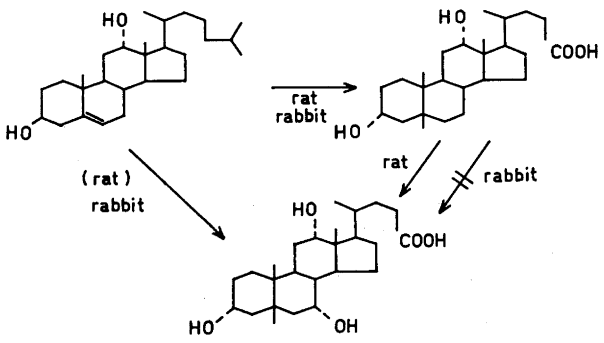

Fig. 3. Summary of the metabolism of 12α-hydroxycholesterol in the bile-fistula rat and bile-fistula rabbit.

way from 12α-hydroxycholesterol to cholic acid that does not entail the intermediary formation of deoxycholic acid. In the rabbit, however, this 7α-hydroxylase is lacking and the results obtained show that there obviously exists such a pathway in this species. The yield of cholic acid from 12α-hydroxycholesterol in the rabbit was rather low, about 10 % of the excreted isotope, while the main product was deoxycholic acid. The present experiments show that the rabbit liver is capable of converting 12α-hydroxycholesterol to cholic acid but do not form any basis for conclusions concerning the possibility that 12α-hydroxycholesterol might be an intermediate in the conversion of cholesterol to cholic acid. The rather low yield of cholic acid does not necessarily mean that this compound is no intermediate, as the metabolism of an intraperitoneally administered compound might not be comparable to that of one formed *in situ*.

Further work is needed to clarify the role of 12α-hydroxycholesterol in bile acid biogenesis.

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The Preparation of *gem* Dithiols under Mild Conditions

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gem Dithiols have been prepared from carbonyl compounds and hydrogen sulphide under pressure [1], from dibenzylketone and hydrogen sulphide in ethanol at ordinary pressure using hydrogen chloride as a catalyst [2] and by the reduction of duplodithioacetone with sodium in liquid ammonia [3].

During the study of duplodithioketones it was found that ketimines easily add hydrogen sulphide in ether solution at temperatures between $-40°$ and 0°C. After acidifying with dilute hydrochloric acid and extracting the amine hydrochloride with water, *gem* dithiols could be obtained, sometimes in good yields.

In some instances it is not necessary to prepare the ketimine. It is quite sufficient to add the amine to an ether solution of the ketone and pass hydrogen sulphide into the solution at low temperature. The yields are improved if anhydrous potassium carbonate is added to the reaction mixture in order to remove the water formed during the reaction. In both cases the products seem to be quite pure, byproducts with higher boiling points being absent. The products give a positive test for *gem* dithiols with lead acetate in ethanol [1] and a positive test for sulfhydryl with sodium nitroprusside. Infrared absorbtion shows a strong absorbtion maximum at 3.96 – 3.98 μ.

Experimental: (A) *Gem* Dithiols from ketimines. 30 g pentylidene-(3)-butylamine [4] was dissolved in 200 ml dry ether and hydrogen sulphide was passed into the solution at $-30°$C. The reaction mixture became cloudy and a crystalline compound separated. The absorbtion of hydrogen sulphide was very rapid; when absorbtion at $-30°$ ceased the temperature was allowed to rise to 0° and hydrogen sulphide was bubbled through the solution for a further 5 h. The flask and contents were cooled to $-20°$ and 5 M hydrochloric acid was slowly added with stirring until the aqueous phase gave an acid reaction. The ether phase was separated, the aqueous phase extracted with ether and the