The Enzymic Formation of 7α-Hydroxycholesterol from Cholesterol in Rat Liver Homogenates

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HENRY DANIELSSON and KURT EINARSSON

Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

The conversion of cholesterol to bile acids has been studied extensively but the individual steps in the formation of the primary bile acids have not yet been completely established. On basis of studies of the metabolism *in vivo* and *in vitro* of cholesterol and different hypothetical intermediates a scheme of the sequence of reactions in cholic acid formation has been proposed.[1] This scheme suggests that cholesterol is transformed into 7α-hydroxycholesterol which in turn is hydroxylated in the 12α-position to yield cholest-5-ene-3β,7α,12α-triol. The latter compound is then converted in several steps into 5β-cholestane-3α,7α,12α-triol. The ready conversion of 5β-cholestane-3α,7α,12α-triol into cholic acid has been demonstrated and the mechanism of this reaction has been elucidated in part.[1] Recent work by Mendelsohn and Staple[2] has provided strong support for the proposed scheme. These authors have demonstrated the formation of 5β-cholestane-3α,7α,12α-triol from cholesterol in 20 000 × *g* supernatant fluid of rat liver homogenates. The steps proposed to occur in the conversion of cholesterol to 5β-cholestane-3α,7α,12α-triol have not yet been demonstrated experimentally.

Earlier attempts to show the formation of 7α-hydroxycholesterol from cholesterol were unsuccessful mainly because the labeled cholesterol added to the incubations was autoxidized in part yielding among other products 7α-hydroxycholesterol and 7β-hydroxycholesterol.[3] The presence of autoxidation during incubations of cholesterol with liver homogenates thus made it difficult to establish any enzymic formation of 7α-hydroxycholesterol. Using microgram quantities of 4-^{14}C-cholesterol and a thin layer chromatographic method for analysis which requires very small amounts of radioactivity it has now been possible to demonstrate the enzymic conversion of cholesterol into 7α-hydroxycholesterol in rat liver homogenates.

Experimental. White male rats of the Sprague-Dawley strain weighing 150–200 g were used. Homogenates, 20 % (liver wet weight/volume), were prepared in a modified Bucher medium[4] with a Potter-Elvehjem type homogenizer using a loose-fitting teflon pestle. Differential centrifugation of the homogenate was performed in the usual manner. Cholesterol-4-^{14}C (Radiochemical Centre, Amersham, England; 60 μC/mg) was chromatographed on a column of aluminum oxide, grade I (Woelm, Eschwege, W.-Germany) prior to use. The labeled cholesterol, 0.4–0.8 μC, was added to the incubation mixtures in acetone solution giving a final acetone concentration of 1 % in the incubation mixtures (*cf.* Ref.[5]). NADH and NADPH were obtained from the Sigma Chemical Company, St. Louis, Mo. Incubations were run aerobically for 30 min at 38°. Extraction of the incubation mixtures was made with 20 volumes of chloroform/methanol 2:1. The extract was washed once with 0.2 volumes of a 0.9 % solution of sodium chloride. The chloroform phase was evaporated to dryness under reduced pressure and the residue chromatographed on a 4.5 g column of Hostalene GW (Farbwerke Hoechst, Frankfurt am Main, W.-Germany) using phase system I.[6] All radioactive material eluted before the unchanged cholesterol was combined and taken to dryness. The residue was dissolved in a small amount of acetone and an aliquot was subjected to thin layer chromatography using benzene/ethyl acetate 3:7 as moving phase. Silica Gel G (Merck, A.G., Darmstadt, W.-Germany) and glass plates (0.4 × 20 × 20 cm) were used. The samples were applied on the plates as bands and the reference compounds (*cf.* Ref.[3]) as single spots. The reference compounds were visualized by spraying with concentrated sulfuric acid while covering with a glass plate the areas of the chromatoplate which contained the biological samples. These areas were then divided into zones according to the positions of the reference compounds, the zones were collected and extracted with methanol. Aliquots of the methanol extracts were assayed for radioactivity in a gas-flow counter.

Results. Incubation of the different subcellular fractions with cholesterol under the conditions described above revealed that the conversion of cholesterol to 7α-hydroxycholesterol was effected by the microsomal fraction (*cf.* Table 1). Addition of 100 000 × *g* supernatant fluid increased the yield of 7α-hydroxycholesterol about three-fold whereas no stimulation was observed upon addition of NADH or

Table 1. Conversion of 4-^{14}C-cholesterol to 7α-hydroxycholesterol in fractions of rat liver homogenates.

The amounts of enzyme fractions used were: 5 000 × *g* supernatant (homogenate centrifuged 10 min at 800 × *g* and then 10 min at 5 000 × *g*), 3 ml; 20 000 × *g* supernatant (5 000 × *g* supernatant centrifuged 10 min at 20 000 × *g*), 3 ml; microsomes (20 000 × *g* supernatant centrifuged 1 or 2 h at 100 000 × *g*), 2 ml corresponding to 3 ml of 20 000 × *g* supernatant; 100 000 × *g* supernatant, 3 ml.

Enzyme fraction	7α-hydroxycholesterol formed, in %
5000 × *g* supernatant	1.9
20 000 × *g* supernatant	1.6
Microsomes, 1 h	0.1
100 000 × *g* supernatant, 1 h	0.5
Microsomes, 1 h + 100 000 × *g* supernatant, 1 h	1.1
Microsomes, 2 h	0.5
Microsomes, 2 h + 1 μmole NADH	0.4
Microsomes, 2 h + 1 μmole NADPH	0.6
100 000 × *g* supernatant, 2 h	0.1
Microsomes, 2 h + 100 000 × *g* supernatant, 2 h	1.4

NADPH. Fig. 1 shows a thin layer chromatogram of the more polar products formed in an incubation of cholesterol with microsomes and 100 000 × *g* supernatant fluid. The main radioactive peak has the same R_F-value as 7α-hydroxycholesterol and the identity of the labeled material with 7α-hydroxycholesterol was established by crystallization to constant specific acitivity after addition of unlabeled 7α-hydroxycholesterol. Worth noting is that only small amounts of radioactivity were present in the areas corresponding to cholestane-3β,5α,6β-triol, 7β-hydroxycholesterol and 7-ketocholesterol, thus excluding the possibility that the 7α-hydroxycholesterol had been formed by non-enzymic oxidation. The identity of the radioactive material with mobility similar to that of 26-hydroxycholesterol has not been established. The total amount of more

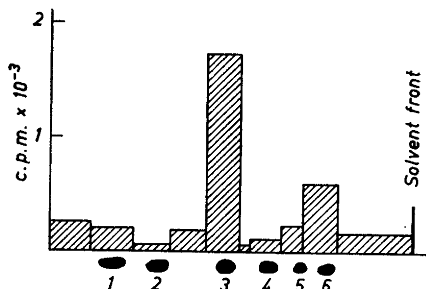

Fig. 1. Thin layer chromatogram of the more polar products formed in an incubation of 4-^{14}C-cholesterol with microsomes + 100 000 × *g* supernatant. Reference compounds: 1 = cholestane-3β,5α,6β-triol; 2 = 5β-cholestane-3α,7α,12α-triol; 3 = 7α-hydroxycholesterol; 4 = 7β-hydroxycholesterol; 5 = 7-ketocholesterol; 6 = 26-hydroxycholesterol.

polar products formed from cholesterol in incubations with microsomes and 100 000 × *g* supernatant fluid varied between 3 and 8 %.

The possible function of this 7α-hydroxylating system in bile acid biogenesis is now being studied.

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1. Danielsson, H. in Paoletti, R. and Kritchevsky, D. *Advances in Lipid Research*, Academic Press, New York 1963, Vol. 1, p. 335 ff.
2. Mendelsohn, D. and Staple, E. *Biochemistry* **2** (1963) 577.
3. Danielsson, H. *Acta Chem. Scand.* **14** (1960) 846.
4. Bergström, S. and Gloor, U. *Acta Chem. Scand.* **9** (1955) 34.
5. Avigan, J., Goodman, D. S. and Steinberg, D. *J. Biol. Chem.* **238** (1963) 1283.
6. Danielsson, H. *Biochim. Biophys. Acta* **27** (1958) 401.

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