On the Oxidation of Cholesterol by Blood *in vitro*

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Although the conversion of cholesterol to bile acids by the liver has been studied extensively [1], very little work has been done on the oxidation of cholesterol by extrahepatic tissue. Meier *et al.*[2] have reported that slices of rat kidney, spleen, adrenals and testes convert the terminal methyl group of 26-^{14}C-cholesterol to $^{14}CO_2$ and suggested that the oxidation by adrenals and testes was directed toward the formation of C-19 and C-21 steroid hormones. Extrahepatic oxidation of cholesterol could be of considerable importance, since by supplying the liver with intermediates in bile acid formation, it would influence the conversion of cholesterol to bile acids.

The observation by Lovelock ** that the cholesterol level in blood, as measured by the Liebermann-Burchard reaction, decreased during the aerobic incubation of blood prompted this investigation of the metabolism of cholesterol by blood using isotopic and chromatographic techniques.

Experimental. "Red blood cells" (likely contaminated whith white blood cells) were isolated from fresh citrated human blood by centrifuging for 10 min at 2 000 × *g*. The packed cells were washed twice by centrifugation with Ringer's solution containing 0.1 % glucose and suspended in an equal volume of the same solution. Lysed cells were prepared by adding an equal volume of distilled water and letting the mixture stand for 5 min at room temperature.

To 10 ml of washed "red blood cells" or lysed cells there was added 1.0 ml of M/10 tris(hydroxymethyl)aminomethane buffer, pH 7.8 and 0.1 ml of 10 % glucose, 2 000 I.U. of benzyl penicillin, 0.1 ml of 1 % versene and 10 μC (160 μg) of 4-^{14}C-cholesterol (Radiochemical Centre, Amersham, England) as a serum albumin suspension [3]. Incubations were carried out aerobically at 37° for 4 h. At the end of the incubation period, the mixture was added dropwise to 5 volumes of ethanol-ether (3:1). The mixture was filtered and the filtrate evaporated to about 20 ml. The filtrate was diluted with water, acidified and extracted twice with butanol saturated with water. The combined butanol extracts were washed with several small volumes of water until neutral and then taken to dryness. The residue was subjected to reversed phase partition chromatography to separate the cholesterol from cholesterol esters and more polar products, using phase-system I[4]. The more polar products were then separated by chromatography into "acidic", "trihydroxy neutral" and "dihydroxy neutral" fractions (*cf.* Ref.[4]) using phase-system III[4]. Rechromatography of the "acidic fraction" was performed with phase systems described by Norman and Sjövall [5].

Results and discussion. Chromatographic analyses of the labeled products isolated from incubations of 4-^{14}C-cholesterol with whole blood, washed "red blood cells", or lysed "red blood cells" showed a small but easily reproducible conversion to more polar products of 2–3 %. The chromatographic pattern of these products was strikingly similar for all types of incubations. The addition of TPNH *, AMP and nicotinamide to washed cells had no stimulatory effects on the oxidation of cholesterol. Significant amounts of cholesterol esters were obtained only from incubations with whole blood.

Partial identification of the more polar products was carried out on material isolated from an incubation of 90 μC of 4-^{14}C-cholesterol with washed "red blood cells". The chromatogram of the more polar products using phase-system III is shown in Fig. 1. The ratio of the "acidic" (8–20 ml of effluent), the "trihydroxy neutral" (25–50 ml of effluent) and the "dihydroxy neutral" fraction (60–150 ml of effluent) was 1:3:6. This was similar to the ratio observed previously for incubations of 4-^{14}C-cholesterol with boiled liver extracts [4]. The results of this experiment were confirmed by analysis of material combined from several small incubations.

The "acidic fraction" was rechromatographed with carrier cholic and chenodeoxy-

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* Abbreviations used are: TPNH, triphosphopyridine nucleotide, reduced form; AMP, adenosine monophosphate.

cholic acid. The radioactivity did not coincide with the titration peaks for the carrier acids, but was distributed in three distinct fractions, one slightly less polar than cholic acid and two less polar than chenodeoxycholic acid. Acidic materials with chromatographic properties similar to the last two fractions have been isolated from autoxidized cholesterol [6].

Results obtained from incubations of cholesterol with liver homogenates [6] indicated that the "trihydroxy neutral" fraction might contain 3β,5α,6β-trihydroxycholestane. Upon recrystallization to constant specific activity with carrier triol, 43 % of this fraction was indeed found to be 3β,5α,6β-trihydroxycholestane. The presence of this triol was confirmed by acetylation of the combined mother liquors and crystals from this experiment. After chromatography on aluminum oxide [6] and recrystallization to constant specific activity, 43 % of the radioactivity was found to be present in the 3,5-diacetate of the triol. The major component (57 %) of the "trihydroxy neutral" fraction was not identified. It did not correspond to any of the known neutral intermediates; after acetylation it was eluted later than the 3,5-diacetate of the triol.

Rechromatography of the "dihydroxy neutral" fraction (75–150 ml of effluent, Fig. 1) showed that the major part of this fraction, about 75 %, had the same elution volume as the epimeric 7-hydroxycholesterols, which can not be separated with this phase system. By isotope dilution it was established that the radioactive material in this peak was a mixture of 7α-, and 7β-hydroxycholesterol; 30 % was identified as 7α-hydroxycholesterol (by crystallization to constant specific activity of the free sterol and of the diacetate) and 74 % as 7β-hydroxycholesterol (by recrystallization of this diacetate and dibenzoate). The remaining 25 % of the "dihydroxy neutral" fraction which was eluted after the epimeric 7-hydroxycholesterols was not identified.

Of the total amount of cholesterol converted to more polar products 65 % was accounted for as 3β,5α,6β-trihydroxycholestane and 7α-, and 7β-hydroxycholesterol. These compounds, together with 7-ketocholesterol, have been shown to be the major autoxidation products of cholesterol (*cf.* Ref.[6]). In spite of the slow oxidation of cholesterol by blood as compared to liver [5], the red and/or the white blood cells could

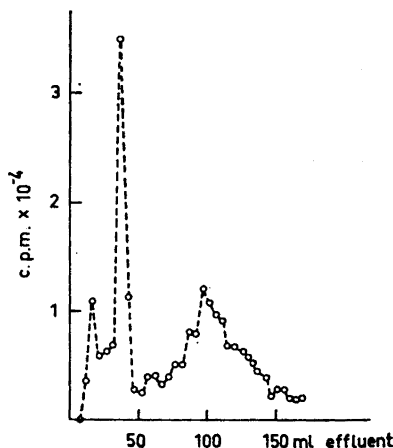

Fig. 1. Rechromatography on phase system III of the more polar fraction obtained from incubation of 90 μC 4-^{14}C-cholesterol with washed "red blood cells". Column: 4.5 g hydrophobic Hyflo Supercel.

play a rôle in the conversion of cholesterol to bile acids if part of the 7α-hydroxycholesterol and of the remaining 35 % of the more polar fraction were formed by enzymic reactions. It appears difficult, however, to establish if this is the case or not.

The present study emphasizes the difficulty of distinguishing enzymic from autoxidation products when microgram quantities of cholesterol are employed in incubations of long duration.

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