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ESTERIFICATION, LIPOPROTEIN BINDING AND EXCRETION OF THE 14β-STEREOISOMER OF CHOLESTEROL

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SUMMARY

Labelled 14β-cholest-5-en-3β-ol (14β-[^{3}H]-cholesterol) was synthesized. No conversion into cholesterol was observed by incubation of this sterol with rat liver enzymes. Plasma decay rate, biliary excretion and binding to lipoproteins did not differ from those of the natural stereoisomer. However, significantly different Lecithin Cholesterol Acyl Transferase (LCAT) activity in plasma was observed when 14β-cholesterol was compared with cholesterol.

INTRODUCTION

Sterols showing the β configuration of the hydrogen at carbon 14 have been recently synthesized by Anastasia *et al.* [1, 2]. All the prepared compounds of this series show chemicophysical properties strikingly different from those of the corresponding 14α-isomers; e.g. 14β-cholesterol shows a lower melting point (38°C) when compared with that of cholesterol (148°C) and a shorter retention time (0.87 relative to cholesterol); moreover, crystallization of this compound from methanol is achieved only by keeping the solution for a long time at −20°C, showing a much higher solubility than cholesterol, which crystallizes from methanol at room temperature. These observations prompted us to test some aspects of the metabolism of these synthetic sterols. Previous studies [3] on the "*in vitro*" catabolism of 5α,14β-cholest-7-en-3β-ol showed the transformation of this "unnatural" compound by rat liver enzymes into 5α-cholest-7-en-3β-ol, a cholesterol precursor.

The results presented in this paper show that 14β-cholesterol is not transformed into the "natural" 14α-isomer. Other studies were therefore carried out to elucidate liver metabolism, binding to lipoproteins and esterification of this compound in order to make clear the importance of using this unnatural compound as a tool in studies of cholesterol metabolism.

EXPERIMENTAL

Materials. All chemicals were of analytical grade. Instruments and working conditions for radio-g.l.c., GC-MS, t.l.c., radio-chromatoscanning and counting of radioactivity were as previously described [3].

Synthesis of 14β-[^{3}*H*]-*cholesterol.* The synthesis was carried out as described by Anastasia *et al.* [2]. Protonolysis following the hydroboration of 3α,5α-cyclocholest-8(14)-en-6α-ol (0.2 g) was performed with tritiated propionic acid obtained by addition of tritiated water (1.3 mCi/ml; 0.62 ml) to propionic anhydride (35 mmol). Saponification of the obtained propionates and purification of 14β-cholesterol was performed as previously reported [2]. The radiochemical and chemical purity of the compound were checked by t.l.c., radio-g.l.c. and GC–MS. Radiochromatoscan of thin layer plates showed only one radioactive peak at the R_F value of the single spot visualized with sulphuric acid. Radio-g.l.c. showed one major radioactive peak (97%) at the retention time of 14β-cholesterol [2] and one minor peak (3%) at the retention time of cholesterol. Mass spectra of the two peaks in the chromatogram corresponded to those of authentic 14β-cholesterol and cholesterol. The specific activity of the compound was 11.6 mCi/mmol as determined by counting a weighed amount of the crystalline material.

Animal experiments. Male rats of the Charles River strain (150–200 g body weight) kept on a standard Charles River rat diet, were used for the experiments reported below. Two male New Zealand rabbits (2.8–3.1 Kg) fed a standard Charles River diet for rabbits and fasted 24 h before the experiment were used to study the binding of 14β-[^{3}H]-cholesterol to plasma lipoproteins.

Metabolism of 14β-[^{3}*H*]-*cholesterol by rat liver enzymes.* 14β[^{3}H]-cholesterol (11.6 mCi/mmol) was incubated with the 10,000 *g* soluble fraction of rat liver homogenate [4]. Conditions and time of incubation, extraction of the unsaponifiable lipids and determination of conversion into cholesterol were carried out as described previously for other sterols [3].

Plasma decay and biliary excretion of 14β-[^{3}*H*]-*cholesterol.* A suspension of [4-^{14}C]-cholesterol (11.6 mCi/mmol; 4 μCi) and 14β-[^{3}H]-cholesterol (11.6 mCi/mmol; 4 μCi) was prepared in 0.5 ml of saline in the presence of Tween 80 (4 mg/ml). The suspension (0.1 ml) was injected into the jugular vein

of rats under pentobarbital anesthesia. Samples of bile were taken from each animal via a P.E. 10 cannula inserted in the common bile duct and blood samples from a carotid artery at the interval times shown in the figures. The per cent transformation of the labelled sterols into acidic compounds was determined after saponification of the bile [5]. After extraction of neutral lipids followed by acidification, the acidic fraction was extracted and counted. T.l.c. of this fraction (benzene–ethyl acetate; 10:90, by vol.) after esterification with diazomethane showed that the radioactivity was associated with compounds with polarity similar to that of cholic acid used as a standard.

Binding of 14β-[^{3}H]-*cholesterol and* [4-^{14}C]-*cholesterol to plasma lipoproteins in rabbit.* The same suspension (0.5 ml) of the two labelled isomers used in the previous experiment was injected i.v. into the ear of two normal rabbits. Plasma samples were drawn from the ear 30 min and 1, 2, 6, 12, 30 and 48 h after injection of the labelled sterols. Aliquots of plasma were counted for the determination of the radioactivity decay curve. Pools of plasma samples from the two rabbits at different intervals were then subjected to preparative ultracentrifugation for the separation of plasma lipoproteins [6]. Fractions of $d < 1.006$ (VLDL), d 1.006–1.063 (LDL) and d 1.063–1.21 (HDL) were separated and counted to calculate distribution of radioactivity. The cholesterol and protein content of each lipoprotein fraction was determined, respectively, by g.l.c. and by the Lowry method [7].

Esterification by plasma LCAT. The esterification of 14β-[^{3}H]-cholesterol and [4-^{14}C]-cholesterol in plasma samples taken from normal human volunteers was determined according to Stokke and Norum [8] except for the substrate concentration which was 5×10^{-5} M instead of 10^{-7} M. For the determination of the per cent esterification of the two substrates, separation of the esters by t.l.c. (hexane–ether–acetic acid; 70:30:1, by vol.) was performed. Plates were scanned for radioactivity and zones corresponding to the R_F of free and esterified cholesterol were eluted and counted.

Table 1. Incubation of 14β-[^{3}H]-cholesterol with 10,000 *g* supernatant of rat liver homogenate*—Incubation conditions: enzyme proteins 180 mg, substrate 1.5×10^{-6} M, phosphate 0.1 M, NADPH 80 μM, pH 7.4; final vol. 6.5 ml

Substrate	% Conversion to cholesterol†
14β-[^{3}H]-cholesterol‡	3.2
14β-[^{3}H]-cholesterol‡	3.1
5α-[2,4-^{3}H]-cholest-7-en-3β-ol	85
5α-[2,4-^{3}H]-cholest-7-en-3β-ol§	0.2

* Mean of duplicate analyses with the same enzyme preparation; results did differ less than 10% from each other. † Determined after purification as the dibromide [3]. ‡ Containing 3% of [^{3}H]-cholesterol as determined by radiogaschromatography. § Boiled control.

RESULTS

As shown in Table 1 14β-cholesterol is not converted into cholesterol by rat liver enzymes. The activity of the enzyme preparation was tested by determining the conversion into cholesterol of a precursor of cholesterol biosynthesis, i.e. 5α-cholest-7-en-3β-ol tritiated at positions 2 and 4 as previously described [9].

The plasma radioactivity decay curves of 14β-[^{3}H]-cholesterol and [4-^{14}C]-cholesterol are reported in Fig. 1 as the mean values obtained in four animals. The results obtained for the two sterols were not significantly different on the basis of a student's t test. The same is true for biliary excretion (Fig. 2) were the peak of radioactivity is detectable 30 min after injection with either sterol; 87% of tritium and 95% of ^{14}C was found in the acidic fraction of the bile extract indicating a similar side chain degradation of the unnatural sterol when compared to cholesterol.

The plasma decay curves for the two sterols were similar also in rabbits (Fig. 3). Distribution of the

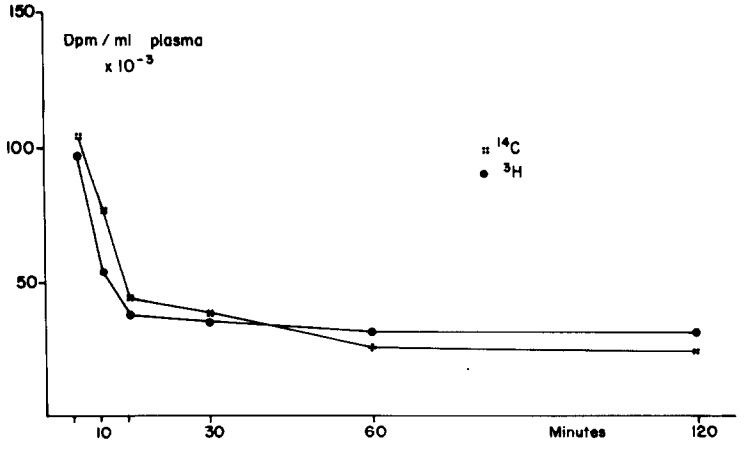

Fig. 1. Plasma decay curves of 14β-[^{3}H]-cholesterol and [4-^{14}C]-cholesterol in rat. Differences between ^{3}H and ^{14}C radioactivity for each point were non significant when analyzed with a student's t test.

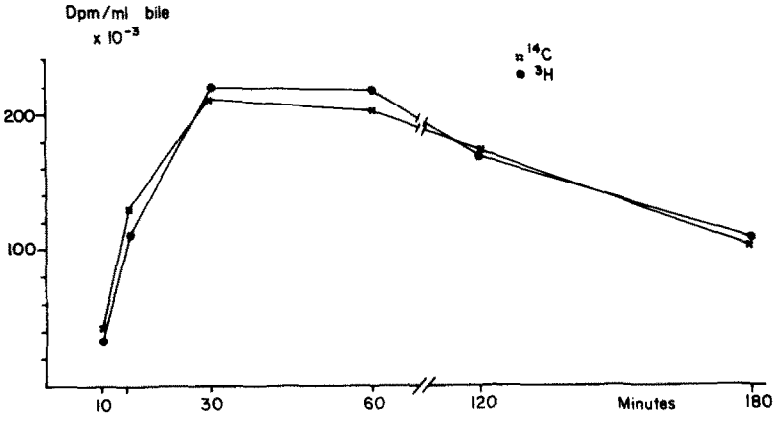

Fig. 2. Bile excretion of 14β-[^{3}H]-cholesterol and [4-^{14}C]-cholesterol in rat. Differences between ^{3}H and ^{14}C radioactivity for each point were non significant when analyzed with a student's *t*-test.

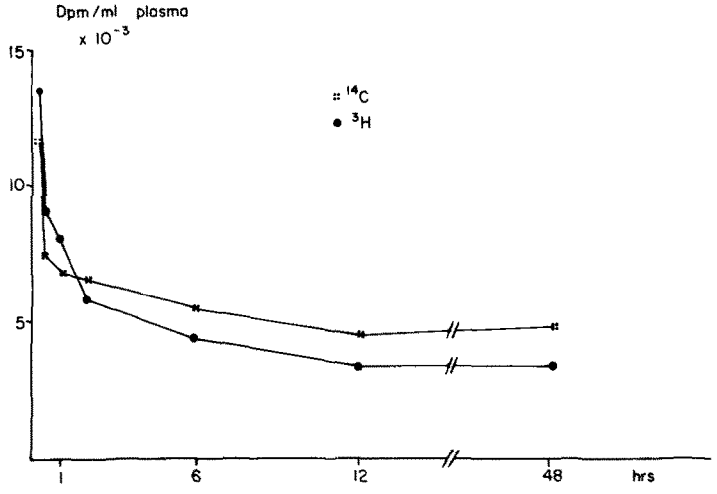

Fig. 3. Plasma decay curves of 14β-[^{3}H]-cholesterol and [4-^{14}C]-cholesterol in rabbit.

two isotopes in ultracentrifugally isolated lipoprotein fractions is shown in Table 2, where no obvious difference in the distribution of the two isotopes is evident.

LCAT activity was determined with 14β-[3H]-cholesterol and [4-^{14}C]-cholesterol as substrates on plasma samples. The enzymatic transformation was significantly higher with the 14β-isomer when compared with the natural one (Table 3). Increased esterification of cholesterol was observed in the presence of 10^{-4} M non labelled 14β-cholesterol.

Table 2. Incorporation of 14β-[^{3}H]-cholesterol and [4-^{14}C]-cholesterol into lipoproteins

Time after administration (h)	% Radioactivity*					
	LDL		VLDL		HDL	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
2	15	15	46	47	39	38
6	9	7	64	67	27	26
24	13	11	60	61	27	28
48	16	12	46	45	38	43

* Considering the total radioactivity found in the three lipoprotein fractions as 100%; figure represent mean value from two rabbits. Values differed less than 10% from each other.

Table 3. Esterification of 14β-[^{3}H]-cholesterol and [4-^{14}C]-cholesterol by human plasma LCAT—Incubation conditions: plasma 0.2 ml, labelled substrate 5×10^{-5} M, albumin 8×10^{-5} M, phosphate buffer 0.1 M, pH 7.1; final vol. 0.4 ml

Subject	% Esterification of*			
	Cholesterol		14β-Cholesterol	
1	1.1		3.6	
2	0.9	1.0 ± 0.08	3.7	3.6 ± 0.1
3	1.0		3.5	
4	1.0		3.7	
2†	2.5 ± 0.1§		—	
2‡	0.8 ± 0.04§		—	

* After subtraction of "blanks" obtained with boiled plasma. † To the incubation medium 0.1 μmol of non labelled 14β-cholesterol were added as a suspension in 0.4 ml of 0.1 M phosphate buffer pH 7.1 containing albumin (0.1 μmol). ‡ To the incubation medium 0.4 ml phosphate buffer containing 0.1 μmol albumin were added. § Mean of three incubation experiments ± S.D.

DISCUSSION

Studies on the metabolism of 14β-cholesterol, an "unnatural" stereoisomer of cholesterol, were carried out by comparing the behaviour of [4-^{14}C]-cholesterol and 14β-[^{3}H]-cholesterol. Our attempt to study the conversion of 14β-cholesterol to cholesterol "*in vitro*" under conditions where other sterols have been shown by us [9] and by others [10] to be converted to cholesterol, failed to demonstrate any conversion of this 14β-isomer of cholesterol to cholesterol. The possibility of conversion to sterols other than cholesterol may also be ruled out on the basis that all the radioactivity present in the starting material as 14β-cholesterol was recovered at the end of the incubation period associated with this compound alone. It may, therefore, be assumed that the metabolic response on the tested systems is representative of the behaviour of 14β-cholesterol and does not depend upon its transformation into cholesterol.

The plasma radioactivity decay curves both in rats and rabbits and the biliary excretion in rats did not show any significant difference between the two stereoisomers. Moreover 14β-cholesterol was converted to acidic compounds in the bile to the same extent as cholesterol.

Molecular models visualize cholesterol as a rigid, flat molecule with all four rings lying in a plane and side chain being free to "float" in space. Similar considerations show that in the 14β-isomer the *cis* C/D ring junction produces a conformational change where ring D now protrudes below the plane of the molecule. However, what effect, if any, this change has on the conformation of the side chain cannot be predicted due to its possibility of floating in the space. If similar changes were to influence the extent of incorporation of 14β-cholesterol into the different lipoproteins, these might have allowed us to draw some empirical conclusions, as a first approximation, regarding size and shape requirements of sterol molecules essential for binding or insertion into lipoproteins. Table 2 shows, however, that the extent of incorporation of radioactivity from both cholesterol and 14β-cholesterol into lipoproteins is similar; the identical behaviour of the two stereoisomers might perhaps indicate a degree of latitude in the conformation requirements necessary for incorporation into lipoproteins.

From the results in Figs 1–3 and in Table 2, it appears that, at least for the aspects studied here, i.e. conversion to bile acids and binding to lipoproteins, 14β-cholesterol follows the fate of cholesterol during its metabolism in liver. In contrast, a significant difference is observed in the "*in vitro*" esterification of 14β-cholesterol by plasma LCAT when compared with that of cholesterol. The LCAT activity was tested by the method of Stokke and Norum [8] where a complete equilibration of a 10^{-7} M exogenously added cholesterol with endogenous cholesterol is observed. In our experiments, either 14β-[^{3}H]-cholesterol or [4-^{14}C]-cholesterol at 5×10^{-5} M concentration were added as substrates to plasma samples of the same subjects. The data show good precision and a 3.5 fold increase of enzyme activity over that observed with cholesterol when 14β-cholesterol was the substrate. Moreover, addition of 10^{-4} M 14β-cholesterol to the incubation medium increases [4-^{14}C]-cholesterol esterification by a factor of 2.5. Therefore, either 14β-cholesterol facilitates the equilibration of exogenous with endogenous cholesterol, or it increases the esterification by favoring the access of the substrate to the active enzyme site.

In view of the critical role played by LCAT in the regulation of lipoprotein and cholesterol metabolism, it would be essential to distinguish between these two possibilities. Clearly, the data at hand do not permit such a distinction. However, experiments are in progress in our laboratory which may elucidate more clearly the role played by 14β-cholesterol in modifying LCAT activity.

REFERENCES

1. Anastasia M., Scala A. and Galli G.: *J. org. Chem.* **41** (1976) 1064–1067.
2. Anastasia M., Fiecchi A. and Scala A.: *J. chem. Soc.* Perkin I. **37** (1976) 370–380.
3. Galli Kienle M., Anastasia M., Cighetti G., Manzocchi A. and Galli G.: *Eur. J. Biochem.* **73** (1977) 1–6.
4. Bucher N. L. R., Mc Garran K., Gould E. and Loud A. V.: *J. biol. Chem.* **234** (1959) 262–267.
5. Eneroth P. and Sjövall J.: In *Methods in Enzymology* (Edited by R. B. Clayton) Academic Press, N.Y., Vol. 15 (1969) p. 237–280.
6. Havel R. J., Eder H. A. and Bragdon A.: *J. clin. Invest.* **34** (1955) 1345–1353.
7. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265–275.
8. Stokke K. T. and Norum K. R.: *Scand. J. clin. Lab. Invest.* **27** (1971) 21–27.
9. Fiecchi A., Galli Kienle M., Scala A., Galli G., Paoletti R. and Grossi Paoletti E.: *J. biol. Chem.* **247** (1972) 5898–5904.
10. Lee W. H., Lutsky B. N. and Schroepfer G. J. Jr.: *J. biol. Chem.* **244** (1969) 5440–5448.